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Research Article

Alternative exon usage selectively determines both tissue distribution and subcellular localization of the acyl-CoA thioesterase 7 gene products

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Abstract. Acyl-CoA thioesterases (ACOTs) catalyze the hydrolysis of acyl-CoAs to free fatty acids and coenzyme A. Recent studies have demonstrated that one gene named *Acot7*, reported to be mainly expressed in brain and testis, is transcribed in several different isoforms by alternative usage of first exons. Strongly decreased levels of ACOT7 activity and protein in both mitochondria and cytosol was reported in patients diagnosed with fatty acid oxidation defects, linking ACOT7 function to regulation of fatty acid oxidation in other tissues. In this study, we have identified five possible first exons in mouse *Acot7* (*Acot7a–e*) and show that all five first exons are transcribed in a tissue-specific manner. Taken together, these data show that the *Acot7* gene is expressed as multiple isoforms in a tissue-specific manner, and that expression in tissues other than brain and testis is likely to play important roles in fatty acid metabolism.

Keywords. Acyl-CoA thioesterase, brain, lipid metabolism, acyl-CoA hydrolase, acyl-CoA, testis.

Introduction

Long-chain acyl-CoA thioesterases (EC 3.1.2.2) (ACOTs) are a group of enzymes that hydrolyze acyl-CoA esters to the free fatty acid and coenzyme A (CoASH). The importance of ACOT enzyme activity is evident on examination of the many different processes involving long chain acyl-CoA esters and free fatty acids. Long-chain acyl-CoA esters play a central role in the biosynthesis of lipids, and degradation of fatty acids *via* β-oxidation. It is now also apparent that, apart from their use as an energy source, long chain acyl-CoA esters play important roles in many other cellular processes, as reviewed [1, 2]. Therefore the regulation of acyl-CoA and free fatty acids is important in intracellular metabolism.
In view of the fact that ACOT enzymes hydrolyze acyl-CoAs to free fatty acids and CoASH, we have speculated that they are functionally linked to lipid metabolism where they regulate intracellular levels of acyl-CoAs and free fatty acids. Several evolutionary unrelated enzymes and protein families of ACOTs

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have now been identified (for review see [3, 4]). The peroxisome proliferator-induced activity in mitochondria and cytosol is due to induction of two protein families [5–7], which were named Type-I and Type-II acyl-CoA thioesterases [8]. The Type-II acyl-CoA thioesterases (now renamed *Acot7* under a new nomenclature system recently introduced [9]) in cytosol was first identified by Miyazawa et al. [5] and two of these isoforms were later cloned from rat [10, 11]. These enzymes were shown to be inducible in liver and testis at both mRNA and protein level by treatment of rats with di-(2-ethylhexyl)phthalate (DEHP), a peroxisome proliferator. These enzymes were shown to have a broad substrate specificity, and could hydrolyze acyl-CoAs from C_6-C_{22}-CoA [5, 8, 12, 13]. There is a very high sequence similarity between the rat, mouse and human ACOT7 sequences, suggesting an important evolutionary conservation of these genes. Brain and testis contain by far the highest ACOT activities, and since 75–90 % of the activity in brain is due to ACOT7 [13], ACOT7 was also originally designated brain acyl-CoA hydrolase (BACH) [11]. In human brain, ACOT7 is expressed in neurons, *e.g.*, pyramidal cells in the cerebral cortex and Purkinje cells in the cerebellum [14]. In developing mouse brain, *Acot7* was shown to be expressed during embryogenesis in association with neuronal differentiation [15], and in adult mouse brain ACOT7 is exclusively localized to neurons [16]. Recently, the genomic structure of the *Acot7* gene family was characterized in both human and mouse [14, 16]. Although these studies showed that several isoforms of *Acot7* are generated from a single gene following alternative splicing of different first exons, with the resulting proteins being expressed mainly in brain and testis, the function of *Acot7* is still unknown in these tissues. However, recently it was demonstrated that some patients diagnosed with fatty acid oxidation defects also has severely decreased ACOT7 expression in fibroblasts, suggesting that ACOT7 may also have important functions outside the brain and testis in regulation of fatty acid oxidation. Since there are almost no data available on the expression of *Acot7* isoforms in tissues besides brain and testis, we performed a careful expression analysis of *Acot7*. First we took a bioinformatics approach to characterize the gene encoding the ACOT7 protein family in mouse. Combined with expression profiling, real-time PCR and cellular localization experiments, we have now identified five alternative first exons encoding isoenzymes with different N-terminal protein sequences that show different cellular localizations and distinct tissue expression patterns.

Materials and methods

Animals and treatments. SV/129 male and female mice were maintained on a normal chow diet (R36, Lactamin, Vadstena, Sweden). All mice were killed by CO_2 asphyxiation, followed by cervical dislocation. Various organs were removed and frozen in liquid nitrogen, and stored at –70°C for preparation of total RNA. Ethical permission was obtained from the Animal Experimental Ethical Committee, Stockholm, for all animal experiments.

Identification of cytosolic and mitochondrial Acot7. A blastn sequence similarity search [17] was carried out against the entries in the mouse Expressed Sequence Tag (EST) database (http://www.ncbi.nlm.nih.gov) using the cDNA sequence for the human cytosolic acyl-CoA thioesterase (now *Acot7_v1*) [13] as a search template. Numerous hits were aligned to the search template and full-length sequences corresponding to mouse *Acot7_v1* (previously called CTE-II or BACHa) were assembled using DNAStar Seqman software (DNAStar). *Acot7_v1* sequences with different 5′ ends were aligned against the sequence in ENSEBML mouse genome database (http://www.ensembl.org) to identify the corresponding alternative first exons. By this strategy, two EST clones with extended 5′ ends (AA466273 and AA103190) were identified. These clones were purchased from UK HPMG Resource Centre (Hinxton, Cambridge, UK) and fully sequenced.

Northern blot analysis. Total RNA was prepared from the various organs using QuickPrep^R total RNA extraction kit (GE Healthcare, Uppsala, Sweden) or Ultraspec RNA kit (Biotecx Laboratories Inc, Houston, Texas), and Northern blot analysis was carried out as described previously [18] using a ^{32}P-labeled full-length probe for mouse *Acot7_v2* (previously called MTE-II or BACHb) cDNA. All blots were exposed to X-ray film at –70°C.

Antibody preparation and Western blot analysis. A peptide was synthesized based on the deduced amino acid sequence of the C-terminal end of the *Acot7_v1* (Acot7a) cDNA – CKAKRQGHT-EPQP-OH (with a cysteine added at the N-terminal end for coupling of the peptide), the antibody to which will recognize all isoforms of the Acot7 gene family. Rabbits were immunized with 500 µg peptide conjugated to keyhole limpet hemocyanin, emulsified in Freund's complete adjuvant. Booster injections of 250 µg of the peptide-conjugate emulsified in Freund's incomplete adjuvant and bleedings were carried out according to standard protocols. Antibodies were affinity purified using a column with the peptide conjugated to Epoxy-activated Sepharose 6B (GE Healthcare). Western blot analysis was carried out on 100 µg homogenate protein (homogenized in 20 mM Tris-HCl, pH 8.0) or 100 µg mitochondrial and cytosolic protein prepared from various male and female mouse tissues and separated by sodium dodecylsulfate-polyacrylamide electrophoresis (SDS-PAGE) on 12 % gels. Western blotting was performed by electrophoretic transfer of the separated proteins onto nitrocellulose filters (Nitropure, Micron Separations Inc., Westborough, MA) using a Transblot cell (Bio-Rad Laboratories, Hercules, CA). The blots were probed with the anti-ACOT7a antibody and subsequently with horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence (ECL™, GE Healthcare) using X-ray film.

Cell transfection and cellular localization experiments. Oligonucleotides were designed based on the sequence of the full-length cDNA for mouse *Acot7_v1* and *Acot7_v2* for cloning of the open reading frames as fusion proteins with green fluorescent protein (GFP). The full-length cDNAs were amplified from mouse kidney (*Acot7_v1*) and brown adipose tissue (*Acot7_v2*) total RNA using One Step RNA PCR kit (AMV) (Takara Biomedicals). Primers used were as follows: 5′ primers: *Acot7_v1* (amplifies from methionine * in Fig. 1b): 5′-AAGATGTCCGGCCCCACCAC-3′; *Acot7_v2:* 5′-ACAATGAAGCTGCTGGTCGGGACT-3′ and the same 3′ primer for both gene products: 5′-GGGGCTGAGGCTCTGTGTGGC-3′. Products were cloned into the pcDNA3.1/CT-GFP vector (Invitrogen), in-frame with the GFP at the C-terminal end. Sequence analysis was performed using Big Dye Terminator (ABI Prism, PE Biosystems) and was sequenced by Cybergene AB (Huddinge, Sweden).

Table 1. Sequence of Sybrgreen primers used for Q-PCR. The 3′ primer used was identical for each *Acot7* gene product, as this primer is located in the second exon, which is common to all gene products.

Gene product	5′ primer	3′ primer
Acot7_v1	TCCGGCCCCACCACAGACACG	CCACATTGGCATCATCTGGAC
Acot7_v2	AGGGACACGGGACTTTCCCTG	CCACATTGGCATCATCTGGAC
Acot7_v3	CGGGACCTCTGCCTCACTT	CCACATTGGCATCATCTGGAC
Acot7_v4	CATGAGGGCTGTCAGAACCA	CCACATTGGCATCATCTGGAC
Acot7_v5	CTGGGATGCTTTGGTGTCCT	CCACATTGGCATCATCTGGAC
Acot7_v7	CTCCCAGGCTCATTCATTCG	CCACATTGGCATCATCTGGAC
Hprt	GGTGAAAAGGACCTCTCGAAGTG	ATAGTCAAGGGCATATCCAACAACA

Human skin fibroblasts were grown in Eagles Minimum Essential Medium (Sigma, St. Louis, MO), supplemented with 10 % fetal calf serum (Life Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin in an atmosphere of 5 % CO_2. Approximately 1.6×10^6 cells were grown overnight in 60-mm dishes on glass cover-slips and were transfected with 10 µg *Acot7_v1*/CT-GFP and *Acot7_v2*/CT-GFP plasmids using the calcium phosphate method as described, but without staining of the nucleus with Hoescht 33342 [19]. Briefly, transfected cells were grown for 48 h, fixed, and permeabilized with Triton X-100. For mitochondrial localization, cells were preincubated for 30 min at 37°C with 100 nM Mitotracker® Orange CMTMRos (Invitrogen Corp., CA) before permeabilization and analyzed directly. For cytosolic localization, cells were incubated with a rabbit anti-GFP antibody (Molecular Probes, Leiden, The Netherlands) and subsequently with a CY3-conjugated affinity pure donkey anti-rabbit IgG (Jackson ImmunoResearch). Cells were examined in Leica DM IRBE fluorescence microscope, using Hamamatsu C4742–95 camera and C4742–95 Twain Interface software.

Quantitative real-time PCR. Total RNA was isolated from various organs from male SV/129 mice using TRIzol reagent (Invitrogen Corp.). Total RNA was treated with DNase1 (Promega Corp.) and pooled from three animals. cDNA synthesis was carried out on 1 µg pooled total RNA from each tissue, using iScript cDNA synthesis kit (Bio-Rad). Quantitative-PCR (Q-PCR) was performed in an ABI Prism 7000 sequence detection system, using Power Sybrgreen Master Mix (Applied Biosystems). Primers were designed based on the cDNA sequences of the various gene products as outlined in Table 1. An endogenous control of mouse hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) was used. The Q-PCR was run in singleplex in triplicate for each sample. Data were analyzed using the ABI Prism 7000 SDS software, and the average C_T value per triplicate was used to calculate the relative amounts of each mRNA transcript, using the $2^{-\Delta\Delta CT}$ method.

Immunoelectron microscopy. Mice were fixed by perfusion with 3 % paraformaldehyde + 0.1 % glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Specimens were then rinsed in 0.1 M PB, infiltrated with 2.3 M sucrose in 0.1 M PB, placed on aluminum holders, and then frozen in liquid nitrogen. Sectioning was performed according to Tokuyasu [20] at –95°C. To block non-specific binding, the sections were placed on drops of 10 % bovine serum albumin (BSA, fraction V, Sigma) in 0.1 M PB for 2 h. Subsequently, the sections were incubated with the ACOT7a primary antibody diluted 1:100 in 0.1 M PB containing 0.1 % BSA (PBB) overnight in a humidified chamber at room temperature. Normal rabbit serum was used as control. The sections were thoroughly washed in PBB and bound antibodies were detected with protein A coated with 10-nm gold. The sections were then washed, fixed in 2 % glutaraldehyde in 0.1 M PB for 10 min, washed in distilled water, and embedded in 2 % methyl cellulose containing 0.1 % uranyl acetate [21]. The grids were examined in a Leo 906 (Oberkochen, Germany) electron microscope at 80 kV.

Results

The *Acot7* gene in mouse is located on chromosome 4 E2, and it was recently shown that the mouse and human *Acot7* isoforms are encoded for by a single gene by alternative use of different first exons, resulting in proteins with different N-terminal ends [14]. The open reading frames are encoded for by nine exons, with exons 2–9 being identical for all isoenzymes. Four different alternative first exons were identified in the human gene, and three alternative first exons were identified in the mouse gene [14]. In an attempt to elucidate the genomic organization of the gene encoding Acot7 in mouse, we initially searched the mouse EST database to identify any further variations in the 5′ region. Based on the database searches we identified two further possible first exons, as shown in Figure 1a, that would result in proteins with the N-terminal amino acid sequences as outlined in Figure 1b. The remaining exons 2–9 were identical in all five gene products. The N-terminal amino acid sequence of one of these variants conforms to a mitochondrial targeting sequence (ACOT7b), whereas the other four isoforms encode putative cytosolic enzymes, as described below. A recent change in nomenclature has been applied to acyl-CoA thioesterases and for the purpose of clarity, the new nomenclature is given in Table 2, as there is a different nomenclature for gene and protein. Proteins in mouse are designated with a lower case suffix (*e.g.*, ACOT7a) based on exon number, whereas the gene is designated as variant, *e.g.*, *Acot1_variant 1* (*Acot7_v1*), *etc.*

Identification and mapping of alternative first exons in the *Acot7a* gene. Previous work has shown that the major isoform of the Acot7 gene expressed in brain and testis is the cytosolic *Acot7_v1* (previously known as CTE-II or BACHa) [10, 13]. To characterize the mouse gene encoding the *Acot7_v1*, searches were carried out in mouse EST databases for different 5′ ends of Acot7. The EST sequences corresponding to

Table 2. Protein and gene nomenclature for ACOT7 isoenzymes. The exon encoding the various gene products is given. * and # refer to methionines in Fig. 1b.

Protein nomenclature	Gene nomenclature	Exon number
ACOT7a *meth	*Acot7_v1*	Ia
ACOT7g #meth	*Acot7_v7*	Ia
ACOT7b	*Acot7_v2*	Ib
ACOT7c	*Acot7_v3*	Ic
ACOT7d	*Acot7_v4*	Id
ACOT7e	*Acot7_v5*	Ie

the different first exons were mapped and the genomic position was also mapped by blast alignments against the mouse genome sequence. This first exon is located most 5′ in the genomic sequence and is denoted Exon 1a (Fig. 1a). The mouse *Acot7_v1*cDNA sequence was assembled using overlapping EST sequences. Interestingly, the ESTs could be divided into two groups, one group of ESTs that would encode a shorter protein of 338 amino acids with a calculated mass of 37.6 kDa. The deduced amino acid sequence of the shorter ACOT7a starts Met-Ser-Gly, and contains 15 amino acids encoded for by the first exon (as shown in Fig. 1a and b, with the start methionine indicated by an asterisk). However, the second group of ESTs contained longer 5′ sequences, and the EST containing the longest 5′ sequence (accession no. CA463703) encodes another 41 amino acids, resulting in a protein of 379 amino acids with a calculated molecular mass of 41.6 kDa, with two further in-frame methionines (Fig. 1a, b). Interestingly, we found four ESTs that encode the longer ACOT protein, named ACOT7g (start methionine indicated by #) (accession nos. CA463703, CA4644199, CB273453 and BY090855), three of which were derived from testis and one from mixed tissues including testis, respectively. In an elegant study, Takagi et al. [22] showed that exon 1a contains two tandemly located initiator elements between the second and third ATG codons that function as transcription start sites, which could explain the presence of the short and long *Acot7_v1* (ACOT7a) and *Acot_v7* (ACOT7g) mRNAs. Notably, all detected ESTs from brain encode the shorter Acot7a protein (methionine *), suggesting that the first exon is differently transcribed in different tissues. This was confirmed using Q-PCR, which shows that all mRNA expressed in testis encodes *Acot7_v7 (*ACOT7g) (see later). This expression pattern is also in agreement with the Western blot data, showing strong expression of two bands in testis (see Fig. 2b). Of the aligned blast hits, a further 8 ESTs with extended 5′ sequences of varying lengths were assembled into a unique contig. For two of these EST clones (AA466273 and AA103190, purchased from UK HGMP Resource Centre) we determined the complete sequence. Sequence analysis identified 58 amino acids in the 5′ end of these clones that were encoded for by an alternative first exon, denoted exon 1b (Fig. 1a, b), with the remaining 323 amino acids being identical to the mouse Acot7a sequence (encoded for by exons 2–9). Examination of the N-terminal amino acid sequence (PSORT II, http://psort.nibb.ac.jp) revealed that it possessed the characteristics of a mitochondrial targeting signal, and correspond to the previously characterized MTE-II or BACHb [8, 14]. The mitochondrial localization was confirmed by GFP experiments (see below). In addition a further EST (accession no. BY134190) was identified that extended further 5′, and encodes 3 more amino acids (Met-Ser-Ala) in the N-terminal end, with the start methionine indicated underlined and in bold face (Fig. 1b). The compiled cDNA sequence for *Acot7_v2* (ACOT7b) contains 1497 bp, of which 1152 bp encodes the open reading frame. The predicted cleavage site of the ACOT7b mitochondrial targeting peptide is indicated in Figure 1b. This putative cleavage site (V-R-T↓R-A) conforms to the 'R-2' class of cleavage sites [(V/A/S)-R-X↓X-(S/A)] described by Schneider et al. [23] and results in a mature ACOT7b protein that is 47 amino acids shorter, with a molecular mass of 37.6 kDa, similar to the Acot7a isoform.

Identification of three further putative first exons. The genome alignment of the assembled mouse *Acot7* EST sequences identified three other possible first exons. The first exon of one of these ESTs (BI409186, expressed in lung) maps 3′ of Exon Ia, and was denoted Exon Id. The splice donor site of Exon Id (AATGAT) differs slightly to the general splice site consensus sequence, and encodes a methionine (underlined) in BI409186 in place of the common arginine (Fig. 1b). This methionine is the start methionine of BI409186. The splice donor sites of the other isoforms conform more strictly to the general consensus for splice donor sites (AAG, GAG, CCG), all of which encode an arginine (Fig. 1b). Another exception is Exon Ic, which contains an in-frame stop codon in the area of the putative first alternative exon, and therefore the open reading frame starts with the first methionine in Exon II common to all isoenzymes. Three ESTs were identified, CA535335 and CK333208 isolated from mixed tissues, and AA275842 isolated from kidney, that are encoded by Exon Ic. Kuramochi et al. [16] identified a 5′-RACE cDNA in mouse (called MBACH-K2), resulting in a protein that is 29 amino acids longer in the N-terminal end. We have now identified one EST (CX225643)

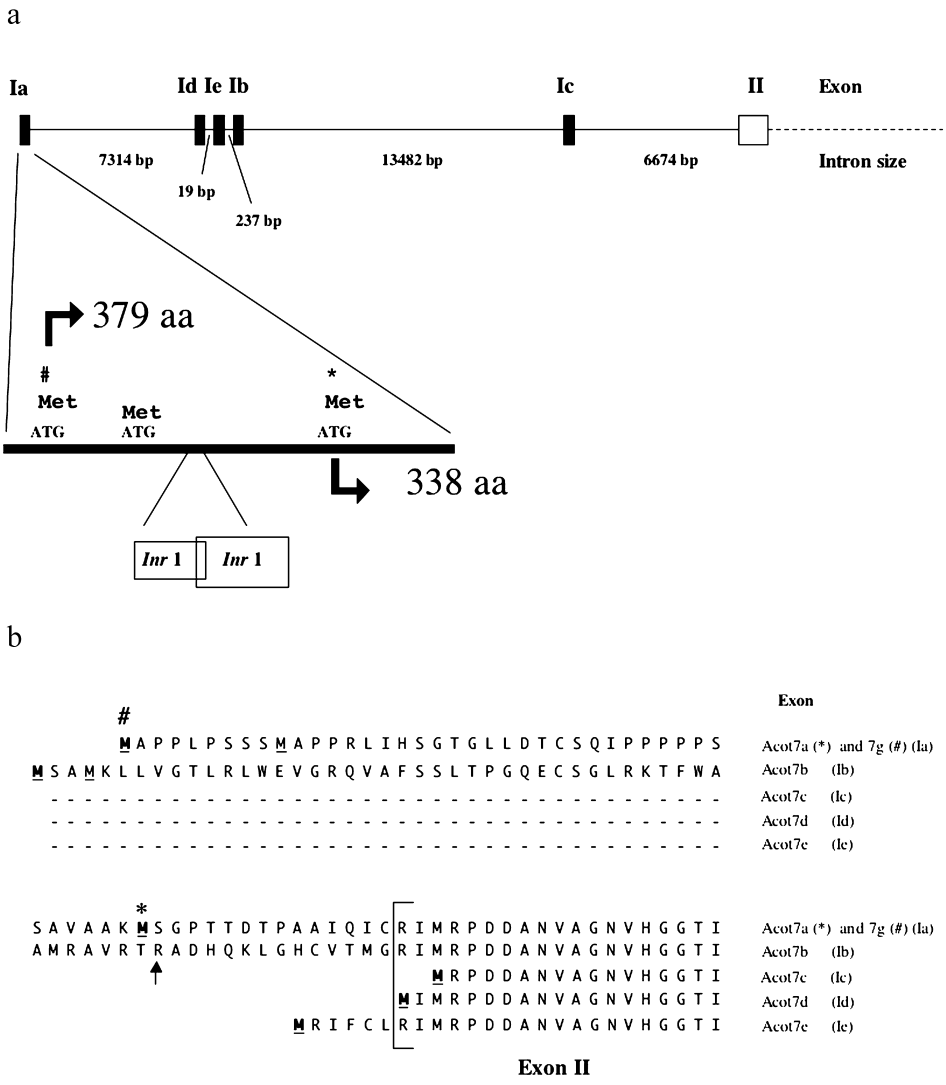

Figure 1. Organization of the 5′ end of the *acyl-CoA thioesterase 7* (*Acot7*) gene. (*a*) The location of the five alternative first exons (Ia–e) is depicted by filled boxes, and Exon II is depicted by an open box. Exon 1a is magnified to shown the location of two tandemly located initiator motif element motifs (*Inr*) that are involved in alternative transcription of Exon Ia. The two alternative start ATG codons are indicated by # (encoding a 379-amino acid protein, ACOT7g) and * (encoding a 338-amino acid protein, ACOT7a). The intron sizes were calculated from the genomic contigs AL772240, AL 671869 and AL 611985. (*b*) Deduced amino acid sequences for the N-terminal ends of the various isoenzymes produced by alternative use of different first exons. The designations of the different first exons (Ia–e) are shown on the far right side. The location of Exon II is indicated by a square bracket. Predicted start methionines are underlined and in bold face. The start methionine for the shorter version of ACOT7a is indicated by an asterisk *, whereas the start methionine for the longer version denoted as ACOT7g is indicated by #. An arrow indicates the predicted cleavage site for the mitochondrial targeting peptide in *Acot7b.*

that corresponds to a differently spliced version of Exon Ic, lacking the in-frame stop codon (see Fig. 1b). A fifth putative first exon (Exon Ie) was identified based on the EST BY346013, which encodes six amino acids (Fig. 1a, b).

The entire gene spans approximately 93.7 kb, of which exons Ia to exon II spans about 28 kb. The sizes of the various first exons are; Ia >241 bp; Ib >252 bp; Ic >222 bp; Id >95 bp; and Ie >129 bp. Thus, the sizes of the transcripts generated by alternative usage of the different first exons are very similar, with only about 125 bp difference between the smallest and largest transcripts. Expression of all five first exons was verified by Q-PCR as described below.

Tissue expression of Acot7 mRNA transcripts. Previous data has shown that in rat [24] and mouse [16], the expression of the *Acot7_v1* transcript and protein is mainly confined to brain and testis. To further investigate the tissue expression of Acot isoenzymes, we examined the mRNA and protein levels in both male and female mouse tissues. Using a radiolabeled full-length cDNA probe for *Acot7_v2* (which will

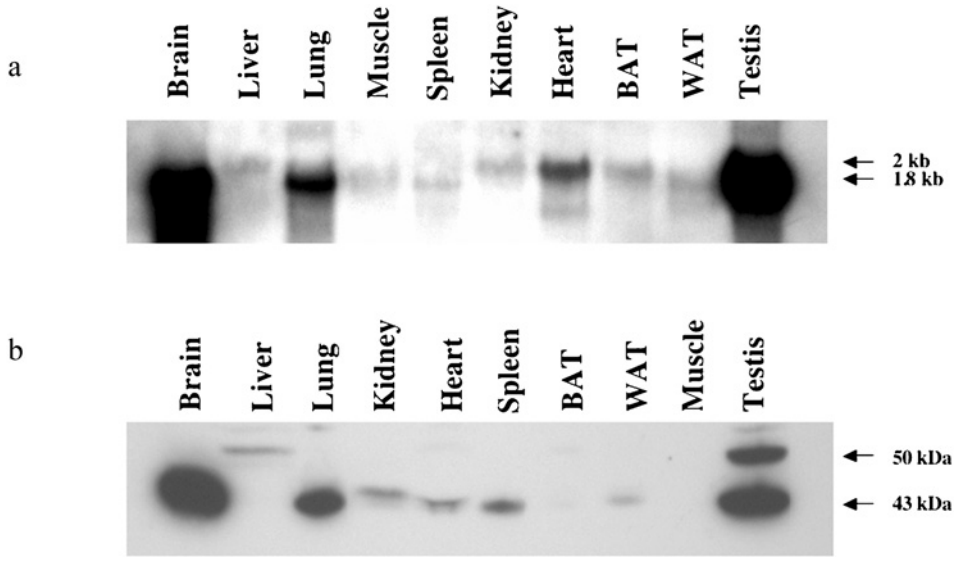

Figure 2. Tissue expression of *Acot7* in male SV/129 mice. (*a*) Total RNA was isolated from various tissues from male SV/129 mice. Northern blot analysis was carried out on 20 μg total RNA using an α-^{32}P-labeled cDNA probe for *Acot7_v2* as described in the Materials and methods. BAT, brown adipose tissue; WAT, white adipose tissue. Sizes of mRNAs are shown. (*b*) Western blot analysis was carried out on 100 μg homogenate protein from various tissues from male SV/129 mice, using an anti-ACOT7 antibody as described in the Materials and methods. BAT, brown adipose tissue; WAT, white adipose tissue. The protein size is shown in kDa.

recognize all Acot7 isoforms), Northern blot analysis identified transcripts of slightly different sizes in all tissues. In male mice, Acot7 expression was highest in testis, followed by brain, but expression was also detected in lung and heart, with weak expression in the other examined tissues: liver, muscle, spleen, kidney, brown (BAT) and white (WAT) adipose tissue (Fig. 2a). A smaller transcript was detected in brain, lung, muscle, WAT and testis, and a slightly larger transcript was detected in liver, kidney, heart and BAT. The larger transcript likely represents both *Acot7_v2* (mitochondrial) and the longer *Acot7_v7* mRNAs, and cannot be resolved by Northern blot analysis.

Western blot analysis of male mouse tissue samples showed strongest expression in brain (a band that migrates as a 43-kDa protein), followed by testis (Fig. 2b). There is evidently a poor correlation between mRNA and protein levels in testis and brain, with much higher protein levels in brain compared to mRNA, while testis contains a higher amount of mRNA than protein (similar to that previously reported in rat and mouse, see [11, 16]), suggesting regulation also at the post-transcriptional level. In addition, strong expression was detected in lung, with weak expression in kidney, heart, spleen and WAT. Notably, the major band detected in liver migrates as a 50-kDa band, similar to a second band detected in testis. The discrepancy between calculated molecular masses (37.6 and 41.6 kDa) and migration on SDS-PAGE (43 and 50 kDa) has been described previously [16], and is most likely due to the non-globular structure of the ACOT7 proteins. Domain analysis suggests that the ACOT7a consists of two homologous domains connected by a hinge region (data not shown).

As the individual isoforms appeared to be differently expressed in a variety to tissues, which could not be resolved by Northern blot analysis, we therefore carried out real-time PCR using primers specific for each of the five isoforms identified. Q-PCR was carried out on pooled total RNA from liver, brain, spleen, lung, kidney, testis and heart. Exon Ia of *Acot7* contains two alternative start methionines, resulting in proteins ACOT7a and ACOT7g, therefore primers were designed to amplify both the *Acot7_v7* (methionine# – ACOT7g) and *Acot7_v1* (methionine* – ACOT7a) versions of this mRNA (methionines indicated in Fig. 1b). Q-PCR was used to amplify both *Acot7_v1* (shorter mRNA) and *Acot7_v7* (longer mRNA) and calculation of the mRNA levels of the individual isoforms of *Acot7_v1* and *Acot7_v7* was carried out as follows: Primers for *Acot7_v1* will recognize both the *Acot7_v1* and the *Acot7_v7* isoforms, whereas primers for *Acot7_v7* are specific only for *Acot7_v7*, therefore if the level of expression of both isoforms was the same in a tissue (as in testis, liver and heart), then all the mRNA related to the *Acot7_v7* (the longer mRNA). If a tissue contained different expression levels of both these isoforms, then the net expression of the *Acot7_v1* was calculated by subtracting the expression level of the *Acot7_v7* from the

Acot7_v1. The *Acot7_v7* is very highly expressed in testis, being 90-fold higher than expression in brain and heart. The *Acot7_v1* is mainly expressed in brain, lung, and spleen, however, in testis all *Acot7a* mRNA is accounted for by *Acot7_v7* (Fig. 3a).
Alternative splicing results in five *Acot7* isoforms, three of which have not previously been characterized, and, as was evident from Northern blot analysis, it appeared that these transcripts were differently expressed in different tissues. Q-PCR was used to examine if the alternative use of the first exons results in transcripts that are expressed in different tissues. The mitochondrial *Acot7_v2* (previously MTE-II or BACHb) has been described in human, but was expressed at only very low levels in human brain compared to *Acot7_v1*, the cytosolic enzyme [14]. Q-PCR from mouse tissues showed that *Acot7_v2* is mainly expressed in heart and lung, with lower levels present in brain and spleen (Fig. 3b). Interestingly, the three newly identified isoforms, *Acot7_v3*, *Acot7_v4* and *Acot7_v5* were all expressed, albeit at a low level compared to *Acot7_v1* or *Acot7_v2*. *Acot7_v3* was highest in spleen, lung, kidney and testis whereas *Acot7_v4* was highest in brain, with detectable levels also in lung (Fig. 3c, d). *Acot7_v5* was highest in spleen, heart and lung (Fig. 3e). It should be emphasized that based on the Q-PCR data, it can be concluded that the *Acot7_v1* and *Acot7_v7* (cytosolic) are the most highly expressed isoforms, followed by the *Acot7_v2* isoform (mitochondrial), and these mRNAs are expressed to a much higher level than the other three isoforms *Acot7_v3*, *_v4* and *_v5*.

Tissue expression of *Acot7* in female mice. In female mice, mRNA expression of *Acot7* isoforms was highest in brain, lung, kidney, heart and ovary (Fig. 4a). In heart and BAT, expression of a larger transcript dominated. Western blot analysis also confirmed these findings, with highest expression in brain, followed by lung, kidney, heart, spleen, ovary and BAT (Fig. 4b). Q-PCR was not carried out on mRNA from female mice.

Expression of ACOT7a and ACOT7b in mitochondrial and cytosolic fractions. Due to the very high sequence similarity between *Acot7* gene products, the anti-Acot7 antibody cannot distinguish between the different isoforms. Therefore to further distinguish between mitochondrial and cytosolic expression of ACOT7 isoforms in mice, we fractionated liver, brain, lung, spleen and heart homogenates into mitochondria and cytosol, and analyzed these fractions by Western blotting (Fig. 5). In mitochondria from liver, a single band of about 43 kDa was detected, and a single band of about 50 kDa was detected in cytosol. This confirms the expression of the longer ACOT7g protein in liver cytosol, whereas liver mitochondria contain ACOT7b protein. We also analyzed mitochondrial and cytosolic fractions prepared from other tissues, which showed that it is mainly the cytosolic enzyme(s) that is/are expressed in lung, spleen and heart. In brain there was a substantial expression of ACOT7 cytosolic proteins and the mitochondrial ACOT7b protein. The ACOT7g protein of 50 kDa was only detected in liver cytosol, with the cytosolic fractions from other tissues containing only the smaller 43-kDa ACOT7 cytosolic proteins (Acot7a, 7c, 7d and 7e). Again, there was a weak correlation between protein levels and mRNA in various tissues. ACOT7b, the mitochondrial isoform, shows highest mRNA levels in heart, whereas low protein levels were detected in heart mitochondria.

Localization of ACOT7a and ACOT7b in cytosol and mitochondria. To examine the intracellular localization of the main ACOT7 isoforms expressed, ACOT7a and ACOT7b, we cloned these proteins in-frame with GFP, which leaves the N-terminal end accessible. We transfected these constructs into human skin fibroblasts. Using immunofluorescence microscopy for detection of a TRITC-labeled secondary antibody to GFP, transfection of the ACOT7a/CT-GFP plasmid showed a strong, diffuse staining, resembling a cytosolic localization (Fig. 6a). Direct analysis of the GFP signal showed that ACOT7b had a distinctive pattern of expression, indicative of a mitochondrial localization (Fig. 6b). This was confirmed using Mitotracker Orange™, which confirmed the structures as mitochondria (Fig. 6c). Sequence analysis of the N-terminal ends of the other ACOT7 isoenzymes identified did not reveal similarity to mitochondrial targeting signals, and are therefore likely to be localized in cytosol.

ACOT7b is highly expressed in late spermatids in testis. As the *Acot7* thioesterases are highly expressed in testis at both mRNA and protein level, we further examined the localization of the ACOT7 protein in this organ. Immunoelectron microscopy showed specific labeling for ACOT7b preferentially in, and in the vicinity of, mitochondria in the late spermatids (Fig. 7a, b). Most of the labeling was detected in the mitochondria around the sperm tail, indicating that ACOT7b protein is highly expressed in testis. Weak labeling was detected in the surrounding cytoplasm, as well as in the cytoplasm of surrounding cells. Labeling with normal serum was homogenously distributed and at the background level indicating an unspecific binding.

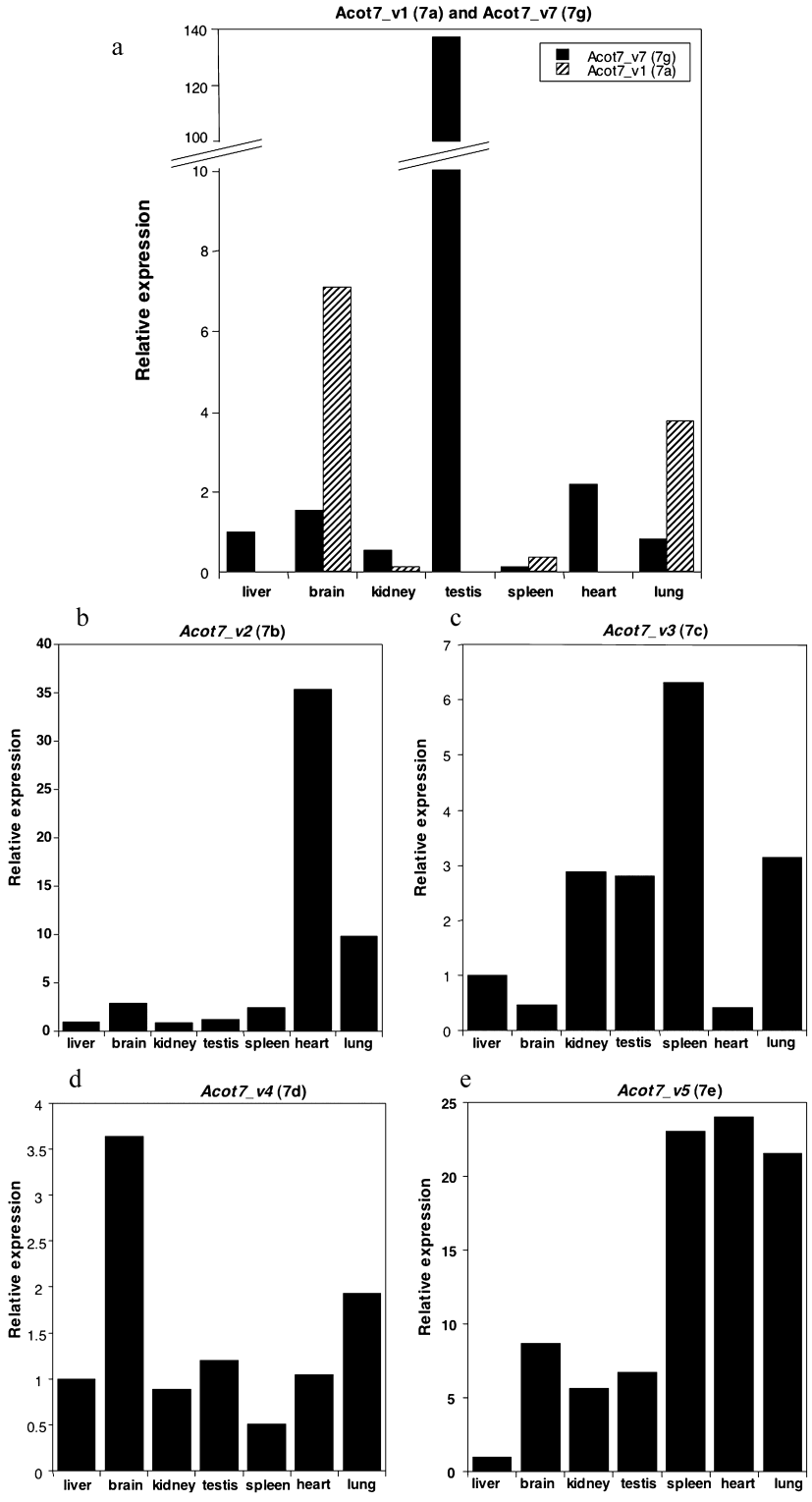

Figure 3. Tissue expression of *Acot7* isoforms in male SV/129 mice using Q-PCR. Tissue expression of *Acot7* isoforms was investigated in mouse tissues using singleplex Q-PCR. Total RNA was prepared from three animals, which was pooled for each tissue and cDNA was prepared from pooled RNA samples. Q-PCR was carried out in triplicate for each tissue, using mouse *Hprt* mRNA as a control. The value for pooled liver for each isoform was set to 1 and the relative expression in tissues calculated. Primers for *Acot7_v1* (7a) also recognize the *Acot7_v7* (7g) as these are encoded by the same exon; however, primers for *Acot7_v7* (7g) are specific for this isoform. Therefore, the relative mRNA levels of *Acot7_v1* (ACOT7a) and *Acot7_v7* (ACOT7g) were calculated by subtracting the values for *Acot7_v7* from the values for *Acot7_v1* where appropriate (as outlined in the Results section). The amount of mRNA relative to liver was calculated using the $2^{-\Delta\Delta CT}$ method. (*a*) *Acot7_v1* (ACOT7a) and *Acot7_v7* (ACOT7g), (*b*) *Acot7_v2* (ACOT7b), (*c*) *Acot7_v3* (ACOT7c), (*d*) *Acot7_v4* (ACOT7d), and (*e*) *Acot7_v5* (ACOT7e).

Discussion

The *Acot7* gene has previously been studied in human and mouse, and shown to be highly expressed in brain and testis. However, only little is known about the expression in other tissues. Our recent finding that screening of patients with undefined fatty acid oxidation deficiencies showed that these deficiencies were associated with markedly decreased ACOT7 activity and protein levels in cytosol and mitochondria [25], suggesting that these enzymes could have more diverse functions than previously envisaged. Molec-

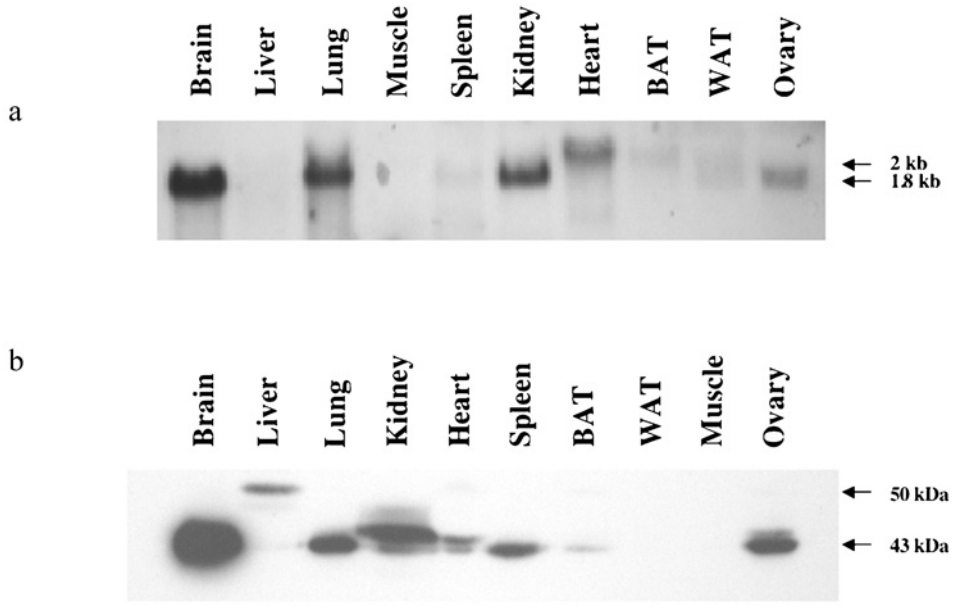

Figure 4. Tissue expression of ACOT7 in female SV/129 mice. (*a*) Total RNA was isolated from various tissues from female SV/129 mice. Northern blot analysis was carried out on 20 μg total RNA using an α-^{32}P-labeled cDNA probe for *Acot7_v2* as described in the Materials and methods. BAT, brown adipose tissue; WAT, white adipose tissue. Sizes of mRNAs are shown. (*b*) Western blot analysis was carried out on 100 μg homogenate protein from various tissues from female SV/129 mice, using an anti-ACOT7 antibody. BAT, brown adipose tissue; WAT, white adipose tissue. The protein size is shown in kDa.

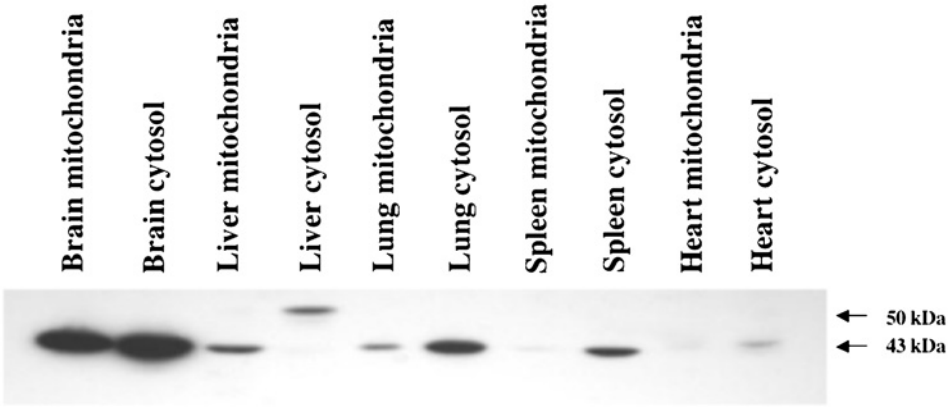

Figure 5. Western blot analysis of cytosolic and mitochondrial fractions from various tissues. Western blot analysis was carried out on 100 μg of cytosolic and mitochondrial fractions prepared from female SV/129 mice using an anti-ACOT7 antibody. The protein size is shown in kDa.

ular characterization showed that the ACOT deficiency was in fact due to decreased expression of ACOT7 in cytosol and mitochondria. Interestingly, ACOT7 activity was responsible for about 80–90 % of the total activity in cytosol and mitochondria in human skin fibroblasts. These data suggest that ACOT7 expression is indeed considerable in other tissues besides brain and testis. The aim of this study was to investigate the complex transcriptional activity of the *Acot7* gene, and to investigate the expression of *Acot7* isoforms in a broader spectrum of tissues in more detail. Here we show that the *Acot7* gene is transcribed into at least five different mRNAs in mouse, due to the presence of five different first exons termed Exons Ia–e. Exon Ia encodes the major brain/testis cytosolic isoenzyme, described previously from rat and human. Using GFP fusion experiments we show that indeed this isoenzyme is localized in cytosol. Interestingly, exon 1a contains three in-frame ATG codons, and it was shown by Takagi et al. [22] that there are two *Inr* elements (*Inr 1* and *Inr 2*) located in tandem between the second and third ATG that can mediate transcription. Translation from ATG 3 would result in a protein of 338 amino acids, while translation

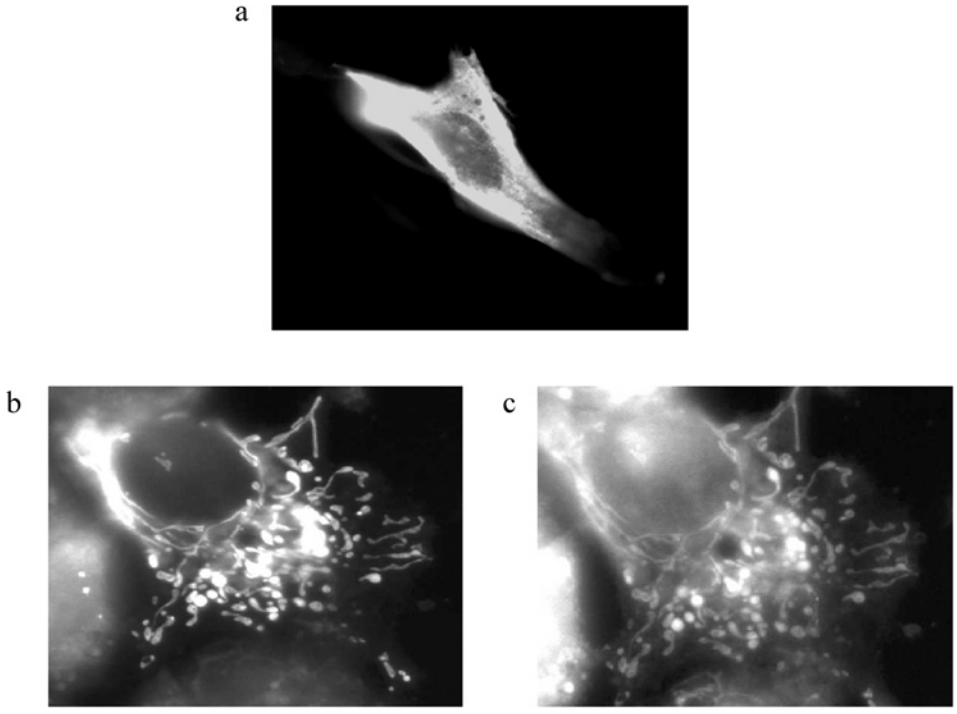

Figure 6. Cellular localization of ACOT7 thioesterases. (*a*) Human skin fibroblasts transfected with ACOT7a/CT-GFP and processed for immunofluorescence microscopy by fixation and permeabilization with 0.5 % Triton X-100. The distribution of Acot7a was examined in fibroblasts using a TRITC-labeled anti-GFP antibody. (*b*) ACOT7b/CT-GFP was examined directly by immunofluorescence microscopy after fixation and permeabilization with 0.5 % Triton X-100. (*c*) Mitotracker Orange™ CMTMRos.

from the first ATG results in a protein of 379 amino acids. Interestingly, exon Ia (encoding the ACOT7g, 379 amino acid protein) is highly expressed in testis, and is the main isoform expressed in liver, kidney and heart. None of the ESTs corresponding to the *Acot7_v7* was expressed in brain tissue, in agreement with the real-time data, showing that it is the *Acot7_v7* that is expressed in testis. Analysis of the N-terminal 41 amino acid extension did not indicate that it would constitute a mitochondrial targeting peptide, and the cytosolic localization of this longer ACOT7g was further strengthened by the apparent cytosolic localization of this 50-kDA protein from fractionation of mouse liver homogenates (Fig. 5) and from GFP-localization experiments [22]. Exon Id does not contain any coding sequence and is translated from exon 2, which results in a protein of 323 amino acids. Exon Ie encodes six amino acids, giving a protein of 329 amino acids in total. Exon Ib encodes the mitochondrial isoenzyme, which was confirmed by GFP-localization experiments. The protein was detected immunologically in liver, lung and testis but mRNA was mainly detected in heart. Finally, splicing of exon Ic results in an in-frame stop codon, which produces a protein of 321 amino acids. We found three ESTs that were all spliced in this manner. However, the MBACH-K2 described by Kuramochi et al. [16] is also encoded by this exon, but was spliced 123 bp further 5′, and therefore lacks the in-frame stop codon. Database searches identified one EST from rat that corresponded to the mouse exon Ic, which was spliced in a similar manner to the mouse gene exon Ic. In contrast, the human gene expresses an exon that is similar to the MBACH-K2 variant, and which in human encodes a second mitochondrial ACOT7, named human ACOT7c [14].

Recent studies have demonstrated a very high degree of sequence conservation of the rat, mouse and human genes, which show >95 % identity at amino acid level. However, these studies have also identified several isoenzymes of ACOTs, some of which are different in human and mouse. Therefore, the reason for the multitude of isoenzymes formed by the alternative use of different first exons is not yet clear. One obvious function is the expression of a mitochondrial isoenzyme that probably plays a role in mitochondrial fatty acid metabolism. The mitochondrial isoenzyme (ACOT7b) was identified and characterized in rat liver as being strongly up-regulated by treatment of rats with peroxisome proliferators [8], which act as ligands for the peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor involved in controlling lipid metabolism [26–29]. The strong regulation of expression of ACOT7b in rat liver by

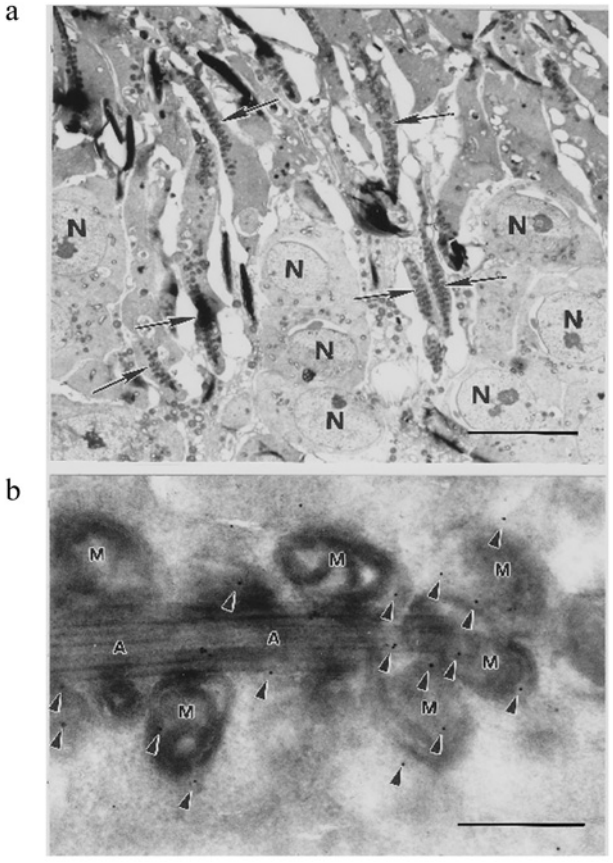

Figure 7. Electron micrograph of mouse testis. (*a*) Early spermatids (N) and late spermatids (arrows) containing high amount of mitochondria. (*b*) Higher magnification showing the distribution of labeling for ACOT7b (arrowheads) in mitochondria (M) and axoneme (A) of a late spermatid. Notably, immunolabeling was only found in spermatids. Bars a=10 μm, b=0.5 μm.

peroxisome proliferators suggests an involvement of ACOT7b in regulation of fatty acid metabolism. Both the cytosolic and mitochondrial enzymes are up-regulated in rat liver following this treatment [10, 11]. However, it was previously shown that the cytosolic ACOT7a enzyme is not up-regulated in liver by treatment of mice with peroxisome proliferators, indicating a species-specific responsiveness [30]. Using both semi-quantitative PCR and size-exclusion chromatography to analyze regulation of *Acot7_v1* and *Acot7_v2* in livers of mice treated with the peroxisome proliferators WY-14,643 or clofibrate, our data show only a minor induction of the mitochondrial ACOT7b in mouse liver (both activity and mRNA) following treatment with WY-14,643 or clofibrate, whereas no up-regulation was detected in the cytosolic fraction (data not shown). Further support for a role of ACOT7b in regulation of fatty acid metabolism stems from our finding that ACOT7b is apparently highly expressed in mitochondria in late spermatids, where these mitochondria are associated to the sperm tail. These mitochondria are small, rounded and dense with condensed matrix and expanded cristae, suggestive of high metabolic activity. Cytosolic ACOT7 is also expressed in spermatids, but not in spermatogonia [22], suggesting that the expression of the *Acot7* gene is restricted to spermatids. In rat, ACOT7b and/or ACOT7a expression is also up-regulated in testis both at the mRNA and protein level after treatment with peroxisome proliferators [10, 12].

Another reason for the existence of numerous alternative first exons may be to mediate tissue-specific expression of *Acot7*. This notion is supported by our data from the Q-PCR and Western blot analyses showing a pronounced tissue-selective expression of different transcripts and proteins. Although previous studies have suggested that ACOT7a is mainly localized in brain and testis in rat [10, 12, 31], our study now shows that these *Acot7* isoenzymes have a much broader tissue expression in mouse than previously thought. It is evident that different ACOT7 isoenzymes are expressed in a tissue-selective manner, for example the 41.6-kDa protein is expressed in liver, testis, and although very weakly, also in heart and BAT. Although expression is much higher in brain and testis than in other tissues, it should be kept in mind that the specific activity of the ACOT7 proteins are generally much higher than the specific activities of most other ACOTs, and would therefore be likely to significantly contribute to the cellular activity in other tissues. This is evident from data obtained with human skin fibroblasts, which showed that about 80 % of the activity in mitochondria and cytosol could be immunoprecipitated using an anti-ACOT7 antibody [25].

Our data, and previous studies, show that there is little correlation between the protein and mRNA expression of *Acot7* in tissues, in particular when comparing the mRNA and protein expression in brain and testis. It has been shown that *Acot7* isoenzymes account for approximately 80–90 % of the ACOT activity in brain [13, 16]. However, mRNA is mainly detected in testis, with lower levels in brain and even lower levels in the other tissues, although protein is highest in brain, followed by testis, lung and lower in the other tissues. It appears that the poorest correlation between mRNA and protein is seen in testis. As discussed above and by Takagi et al. [22], *Acot7* is specifically expressed in spermatids, in which certain mRNAs have been reported to assemble into messenger ribonucleoprotein particles, which temporarily repress translation. Such a post-transcriptional regulation of translation may play important functions under conditions when new mRNA is not being synthesized. Besides PPARα regulation in rat, only little is known about other factors that can regulate transcription of

the *Acot7* gene. It is possible that the different first exons may allow selective regulation of expression by various hormones or other factors. ACOT7 expression was found to be up-regulated in porcine corpus luteum during pregnancy [32], indicating hormonal regulation, and which may also be in line with the pronounced expression seen in mouse ovaries. Recently, the human *Acot7a* promotor was found to be regulated by SREBP-2 *via* a sterol regulatory element (SRE) [33], although the role of this regulation is not yet clear.

The functions of the various ACOT7 isoenzymes are not yet fully clarified. It has been speculated that these enzymes may play a role in lipid synthesis; however, over-expression of the ACOT7a in Chinese hamster ovary (CHO) and C3H 10T1/2 fibroblastic cells did not result in any change in phospholipid synthesis or incorporation of palmitate into various lipid fractions in the cells [10, 34]. Although some cell lines, *e.g.*, CHO cells [10], express high levels of ACOT7a, over-expression of ACOT7a in C3H 10T1/2 fibroblastic cells reduced cell growth. Therefore, it is possible that ACOT7a does not have a general function in regulation of acyl-CoA levels in cytosol, but rather plays a role in cellular signaling. ACOT7a shows high activity towards arachidonoyl-CoA [35], and may therefore play a role in maintaining free arachidonic acid for signaling via arachidonic acid, or for prostaglandin synthesis. Another interesting aspect is that both brain and testis have a blood-tissue barrier. This barrier restricts lipoprotein-mediated uptake of fatty acids from the circulation and therefore brain and testis rely on endogenous fatty acid synthesis, especially 20- and 22-carbon polyunsaturated fatty acids (PUFAs). It may be therefore that Acot7a is involved in synthesis of complex lipids in these tissues. So far, no patients have been identified with mutations linked to any particular disorder, but a derangement of ACOT7 protein has been detected in the hippocampus of patients with mesial temporal lobe epilepsy [36], and decreased levels of ACOT7 protein was detected in fibroblasts from patients with undefined fatty acid oxidation disorders [25].

In conclusion, we have now identified five alternatively spliced *Acot7* thioesterases, which are products of a single gene in mouse. Although these enzymes are likely to have a similar acyl-CoA chain length specificity, similar to the human gene family [14], it is apparent that the different first exons determine selective tissue expression and regulation of expression.

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