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Molecular cloning, gene structure and expression profile of two mouse peroxisomal 3-ketoacyl-CoA thiolase genes

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Abstract

Background: In rats, two peroxisomal *3-ketoacyl-CoA thiolase* genes (A and B) have been cloned, whereas only one *thiolase* gene is found in humans. The aim of this study was thus to clone the different mouse *thiolase* genes in order to study both their tissue expression and their associated enzymatic activity.

Results: In this study, we cloned and characterized two mouse peroxisomal *3-ketoacyl-CoA thiolase* genes (termed *thiolase A* and *B*). Both *thiolase A* and *B* genes contain 12 exons and 11 introns. Using RNA extracted from mouse liver, we cloned the two corresponding cDNAs. *Thiolase A* and *B* cDNAs possess an open reading frame of 1272 nucleotides encoding a protein of 424 amino acids. In the coding sequence, the two *thiolase* genes exhibited ≈97% nucleotide sequence identity and ≈96% identity at the amino acid level. The tissue-specific expression of the two peroxisomal *3-ketoacyl-CoA thiolase* genes was studied in mice. *Thiolase A* mRNA was mainly expressed in liver and intestine, while *thiolase B* mRNA essentially exhibited hepatic expression and weaker levels in kidney, intestine and white adipose tissue. Thiolase A and B expressions in the other tissues such as brain or muscle were very low though these tissues were chiefly involved in peroxisomal disorders. At the enzymatic level, thiolase activity was detected in liver, kidney, intestine and white adipose tissue but no significant difference was observed between these four tissues. Moreover, *thiolase A* and *B* genes were differently induced in liver of mice treated with fenofibrate.

Conclusion: Two mouse *thiolase* genes and cDNAs were cloned. Their corresponding transcripts are mostly expressed in the liver of mice and are differently induced by fenofibrate.

Background

In eukaryotic cells, fatty acyl-CoA β-oxidation systems are mainly located in two organelles, peroxisomes and mitochondria. The major difference between mitochondrial and peroxisomal β-oxidations is their substrate specificity: mitochondria mainly oxidize short, medium, and most long chain fatty acids, while peroxisomes preferentially

oxidize very long chain fatty acids and branched-chain fatty acids [1].

In rodents, two distinct peroxisomal β-oxidation pathways are found to metabolize either straight-chain fatty acids [2,3] or branched-chain fatty acids [4]. Each pathway contains its own enzymes encoded by different genes. Straight-chain fatty acyl-CoAs are catabolized by fatty acyl-CoA oxidase (AOX), peroxisomal L-3-hydroxyacyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (bifunctional enzyme: L-PBE) and peroxisomal 3-ketoacyl-CoA thiolase (PTL). The enzymes involved in the branched-chain fatty acids pathway include branched-chain fatty acyl-CoA oxidase, peroxisomal D-3-hydroxyacyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase (bifunctional enzyme: D-PBE) and sterol carrier protein 2/3-ketoacyl-CoA thiolase (SCP2 /thiolase) also named sterol carrier protein x (SCPx). Humans differ since both very long chain and branched-fatty acids are degraded by D-PBE [5]. L-PBE might not be required for these degradations. Very long chain fatty acids seem to be degraded by PTL as well as by SCPx.

Administration of peroxisome proliferators to rodents results in peroxisomal proliferation and induction of the three peroxisomal enzymes involved in the straight-chain fatty acid β-oxidation i.e. AOX, L-PBE and PTL. In rat liver, the level of these enzymes can be over 20 times as high after treatment with di-(2-ethyl-hexyl)phthalate, a peroxisome proliferator [6]. In contrast, a weak induction of the enzymes of the peroxisomal branched-chain fatty acids system is observed after treatment with clofibrate [7].

In the rat, peroxisomal 3-ketoacyl-CoA thiolase activity is encoded by two distinct genes: *thiolase A* and *thiolase B* [8,9]. The aim of this study was to clone the mouse *thiolase* genes in order to study the tissue expression and associated enzymatic activity. This work will lead to further study of the regulation of their corresponding transcripts, the functionality of the thiolase promoters and the identification of the response elements implicated in their regulation.

We cloned and characterized two mouse peroxisomal *3-ketoacyl-CoA thiolase* genes and their corresponding cDNAs. The tissue distribution of their corresponding transcripts is described in mice: *peroxisomal thiolase A* and *B* transcripts are mainly expressed in liver [preliminary results, [10]]. With the aim of following gene expression main steps, this study was undertaken at the mRNA and enzymatic levels.

Results and discussion

Cloning of two mouse peroxisomal thiolase genes

Genomic DNA extracted from 129SV mouse was digested by different restriction enzymes and analyzed by Southern blotting (figure 1a). The membrane was hybridized with an exon 9 rat thiolase probe common to both A and B isoforms. Each genomic DNA digestion revealed two distinct bands suggesting the existence of two mouse *thiolase* genes. By screening a 129SV mouse genomic DNA library, two distinct sets of clones were identified corresponding to the two different mouse *thiolase* genes. These clones were sequenced and named *mThA* (for mouse *Thiolase A*) and *mThB* (for mouse *Thiolase B*) according to their analogies with their rat counterparts. By comparing these sequences with the database, we identified a genomic clone containing all of the mouse *thiolase B* gene under the accession no AC055818 (Genbank). In this clone, *thiolase B* gene spans from position 72906 (position for the +1 transcription initiation site determined below) to position 81956 (position for the polyA site determined by cloning cDNA, see below). For the *thiolase A* gene, no complete sequence was found in database and therefore, we completely sequenced our genomic clone and deposited it under GenBank accession no AY304542.

According to the Mouse Genome Sequencing Project, we identified part of the mouse *thiolase A* and *B* genes within a contig from chromosome 9 on position f4 (REFSEQ accession no NT_039482). In the rat, the two *thiolase* genes also colocalize on the same chromosome (chromosome 8 on position q32) while in humans, only one peroxisomal *3-ketoacyl-CoA thiolase* gene has been cloned and located onto chromosome 3p22-p23 [11].

Functional analysis

Sequence determination of the two genes showed that they exhibited 97% nucleotide identity in their coding region, whereas introns were relatively different in size and in sequence (Table 1). The same observation is made for the two rat *thiolase* genes [8]. Both mouse genes span roughly 9 kb and contained 12 exons and 11 introns (figure 1b). All of the acceptor-donor sequences for RNA splicing conformed to the GT/AG rule (Table 1) [12].

To determine the transcription initiation sites of the mouse *thiolase* genes, we used the RNase protection method with two antisense RNA probes (specific to each *thiolase* gene) (figure 1c). Thiolase A and B probes were obtained by PCR (as indicated in Methods) with the oligonucleotides indicated by arrows on figures 3a and 3b, respectively. These probes (678 nt for mThA and 521 nt for mThB) extended from the end of exon I (167 nt downstream the ATG codon for the mThA and mThB probes) to 409 nt and 251 nt upstream the ATG initiation codon for *thiolase A* and *B* genes, respectively. The transcription start

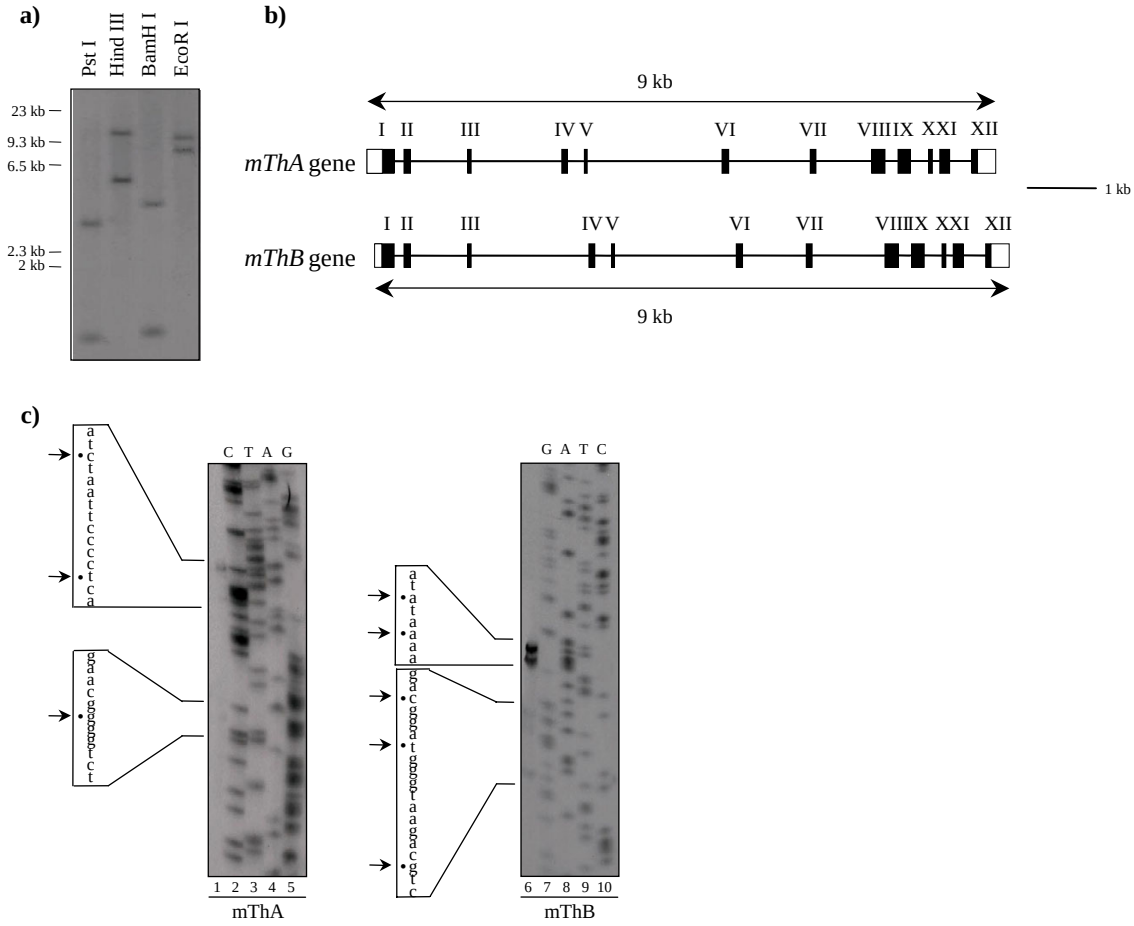

Figure 1

Cloning of the two mouse peroxisomal *thiolase* genes and determination of the transcription initiation sites of each gene. a) Southern blot of a 129SV mouse genomic DNA digested with different restriction enzymes. Membrane was hybridized with an exon 9 rat *thiolase* ^{32}P-labelled cDNA probe. A molecular weight marker DNA is indicated on the left in kb. b) Structural organizations of the mouse *thiolase A* and *B* genes: exon-intron distribution. Exons are numbered from I to XII. Translated sequences are shown as black boxes and untranslated sequences are open boxes. c) Identification of the transcription initiation sites of mouse *thiolase A* and *B* genes by ribonuclease protection assay. RNA probes corresponding to the genomic sequence extending upstream from the exon 1-intron 1 border (probes size: 678 nt and 521 nt for mThA and mThB, respectively) were hybridized to mouse liver RNA (lanes 1 and 6) and digested with RNAses. The protected fragments were analysed on a denaturing polyacrylamide gel (for details, see Methods). Lanes 2–5 and 7–10, DNA sequencing ladder from plasmids containing +1 mThA and +1 mThB probes, respectively. Arrows and dots indicate the different bands corresponding to multiple transcription initiation sites.

sites were determined with liver RNA extracted from mice. The thiolase A probe protected three fragments of 388 nt, 398 nt and 431 nt (figure 1c, lane 1) allowing to localize three transcription initiation sites indicated by dots on figures 1c and 3a. The most intense band corresponds to the largest fragment. For thiolase B, five fragments were protected: 267 nt, 270 nt, 284 nt, 288 nt and 298 nt (figure 1c, lane 6). The 267 nt-protected fragment was considered as the major transcription initiation site and designed as +1 (figure 3b). As control, experiments using the same RNase concentration were performed without RNA hybridization or using yeast tRNA alone for hybridization showing that the probes were totally degraded under our experimental conditions (data not shown).

Using RT-PCR on mouse liver RNA, we cloned two cDNAs, which were completely sequenced and correspond to the *thiolase A* and *B* genes. The 3' end of each *thi-*

Table 1: Exon-intron boundaries of the mouse peroxisomal 3-ketoacyl-CoA thiolase A (upper panel) and B (lower panel) genes[a].

Thiolase A					
Exon		Intron		5' Splice donor	3' Splice acceptor
Number	Length (bp)	Number	Length (bp)		
1	392	1	139	AGGCTTCAAG**gt**gaggcccc...	...cgccctgc**ag**AACACCACCC
2	94	2	804	ATCTCCGTGG**gt**gagctccc...	...ctatctac**ag**GCAATGTTCT
3	58	3	1392	AATTTCTGAG**gt**aactcttt...	...tctgtttc**ag**CGGCATCCCA
4	80	4	213	AACATTGCTG**gt**aagtggtg...	...tttattgc**ag**GTGGCATCAG
5	43	5	1961	TGGCCTGTGG**gt**aagatcgc...	...tcctcctt**ag**GGTGGAGTCC
6	99	6	1161	CTCCTATGGG**gt**gagtgttc...	...tgtgcact**ag**GATGACCTCG
7	81	7	770	CCCAGCAGAA**gt**gagtgtgg...	...gtgctggc**ag**GGCAGCAAGC
8	191	8	149	ACCACGGCTG**gt**gagcgcag...	...cctcccct**ag**GAAACTCCAG
9	180	9	253	CAGAAAGCAG**gt**gaggtggc...	...ttgtctac**ag**GGCTGACTGT
10	56	10	82	TGCAAGTCAG**gt**gagcctgg...	...ttccttcc**ag**GCCGTCTACT
11	146	11	320	GTGGCAGACG**gt**aaggctgc...	...cctttccc**ag**GGCTTACGGC
12	338				
Thiolase B					
Exon		Intron		5' Splice donor	3' Splice acceptor
Number	Length (bp)	Number	Length (bp)		
1	268	1	139	CTGCTTCAAG**gt**gaggcccc...	...cgccctgc**ag**GACACCACCC
2	94	2	788	ATCTCCGTGG**gt**gagctccc...	...ctatccac**ag**GCAATGTGCT
3	58	3	1739	AGTTCCTGAG**gt**aatctctc...	...tctgtttc**ag**CGGCATCCCA
4	80	4	213	AACATTGCTG**gt**aagtggtg...	...tttattgc**ag**GTGGCATCAG
5	43	5	1755	TGGCCTGTGG**gt**aagatcgc...	...ttttcatc**ag**GGTGGAGTCC
6	99	6	911	TTCCTATGGG**gt**gagatatct...	...tgtgcact**ag**GATAACTTCG
7	81	7	1033	CCCAGCAGAA**gt**gagtgtgg...	...gtgctggc**ag**GGCAGCAAGC
8	191	8	149	ACCACGGCTG**gt**gagcgcgg...	...cctcccct**ag**GAAACTCCAG
9	180	9	253	CAGAAAGCAG**gt**gaggtggc...	...ttgtctac**ag**GGCTGACTGT
10	56	10	82	TGCAAGTCAG**gt**gagcctgg...	...ctccttcc**ag**GCCGTCTACT
11	146	11	320	GTGGCAGACG**gt**aaggctgc...	...cctttccc**ag**GGCTTACGGC
12	371				

[a] The sizes of the exons and introns, along with the exon-intron boundary sequences, are shown. Exon sequences are given in capitals and introns in lower case letters. Each intron begins with a GT (in bold) and ends with an AG (in bold).

olase cDNA was determined by 3' RACE (for details see Methods) and the 5' ends were deduced from RNase protection results (figure 1c). These complete cDNAs (1758 bp and 1667 bp for *mThA* and *mThB*, respectively) are presented in figure 2 and referenced in GenBank with accession nos AY273811 (for *mThA*) and AY273812 (for *mThB*).

The mouse *thiolase A* and *B* cDNAs displayed 85% nucleotide identity (figure 2). The translation initiation site was assigned to the first ATG triplet downstream of the major 5' transcription initiation site. The nucleotide context around the ATG codon conformed to the consensus sequence of the eukaryotic translation initiation signal [13]. This start codon allowed the translation of a precursor protein of 424 aa, showing more than 95% protein identity with the rat peroxisomal thiolase proteins (referenced on ncbi server http://www.ncbi.nih.gov with accession numbers: NP_036621 for rThA protein and BAA14107 for rThB protein). Then, these proteins were cleaved into two mature forms of 398 aa (figure 2). Whereas in rat genes the *rThA* mRNA encodes a precursor form 10 aa longer than the protein encoded by the *rThB* mRNA [9]. Nevertheless, after cleavage of the two precursor isoforms, the two thiolase A and B proteins had the same size. The *rThA* mRNA contains two in frame ATG at its 5' end while *mThA* mRNA contains only one ATG codon.

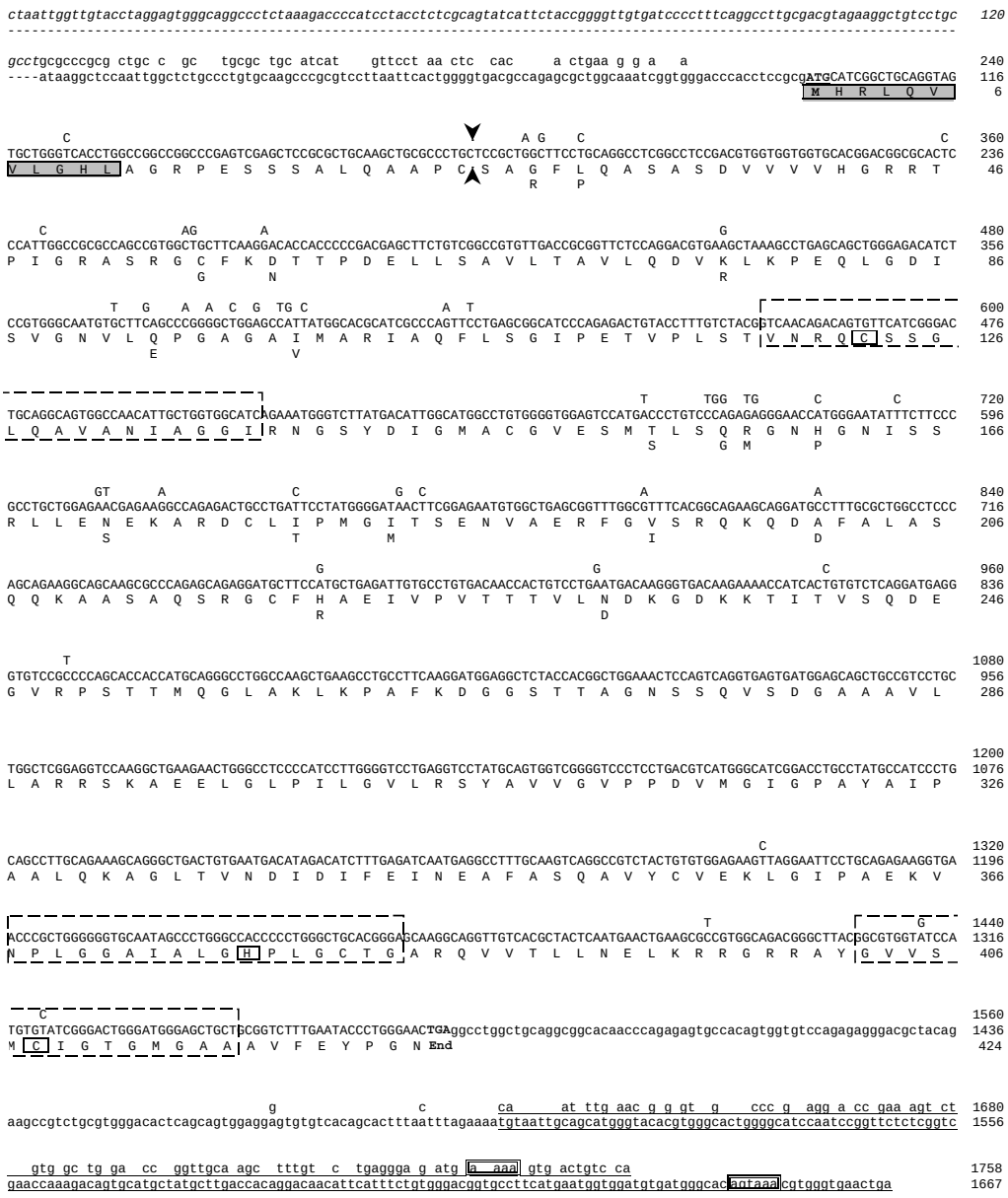

Figure 2

Nucleotide sequence and deduced aa sequence of the *mThB* cDNA and comparison with *mThA* cDNA. The complete nucleotide sequence for *mThB* cDNA and its deduced aa sequence are shown in full. Differences with mThA cDNA are indicated above the mThB sequence, whereas differences in the deduced aa sequence are indicated below. The positions of nucleotides and aa residues are given on the right. Nucleotide 1 (for each cDNA) corresponds to the major transcription initiation site determined by ribonuclease protection assay (figure 1c). The start codon (ATG) is indicated in bold and underlined, and the stop codon (TGA) is in bold and denoted by End. The coding regions are written in capital letters, whereas the untranslated regions are in lower case letters. Dashes (-) correspond to the absence of 5' and 3'-untranslated extension in each cDNA. The 5'-extension of mThA cDNA (124 nt) appears in italics. The underlined sequences correspond to specific probes (used for Northern blot) of *thiolase A* and *B* cDNAs, respectively. The putative polyadenylation signal AGTAAA is double-framed in the two sequences. The putative Peroxisomal Target Signal (PTS 2) is framed and shaded. The three thiolase signature patterns are framed in dotted lanes. The putative catalytic residues (Cys123, His377 and Cys408) are framed. Arrows indicate the putative cleavage site for the mature thiolase proteins. The *thiolase A* and *B* cDNAs have been deposited in the GenBank database: accession nos AY273811 and AY273812, respectively.

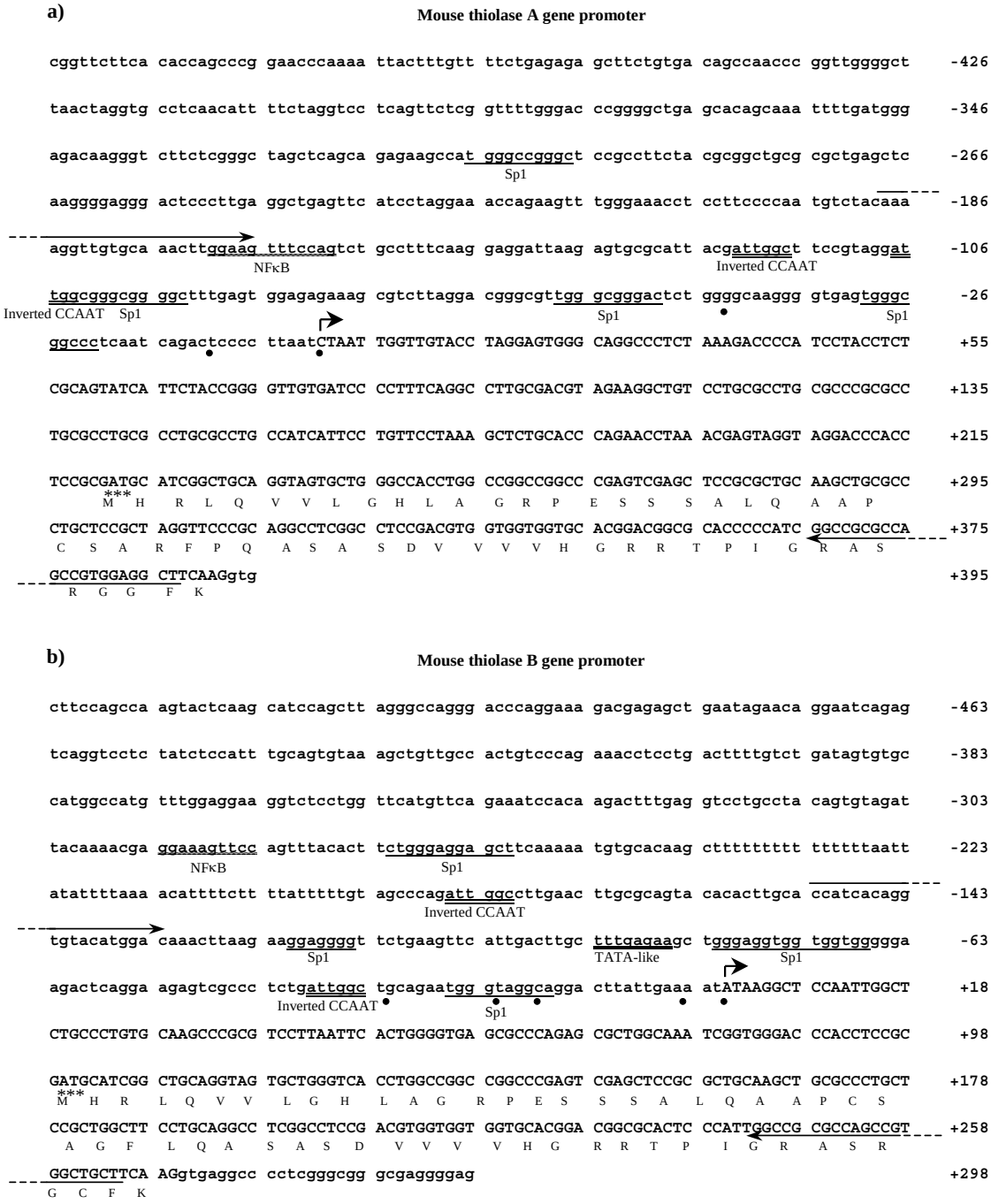

Figure 3

Nucleotide sequences of the 5' upstream region and the 5' end portion of the mouse *thiolase A* (a) and *thiolase B* (b) genes. The exon 1 sequence is written in capital letters and the promoter region and the beginning of intron 1 is in small letters. The predicted aa sequence of the exon 1 is under the coding nucleotide sequence. The arrows indicate oligonucleotides used to prepare probes for RNase protection assay. Nucleotide 1 (designed by the bent arrow) corresponds to the major transcription initiation site determined by RNase protection assay (see figure 1c). The multiple transcription initiation sites predicted by RNase protection are indicated by dots. Numbers on the right of figure are the nucleotide position relative to +1. The putative initiator methionine codon is asterisked. Potential GC boxes (Sp1 binding sites) are indicated by underline, complement of CCAAT box by double underline, and homolog of NFκB binding sites by double wavy line.

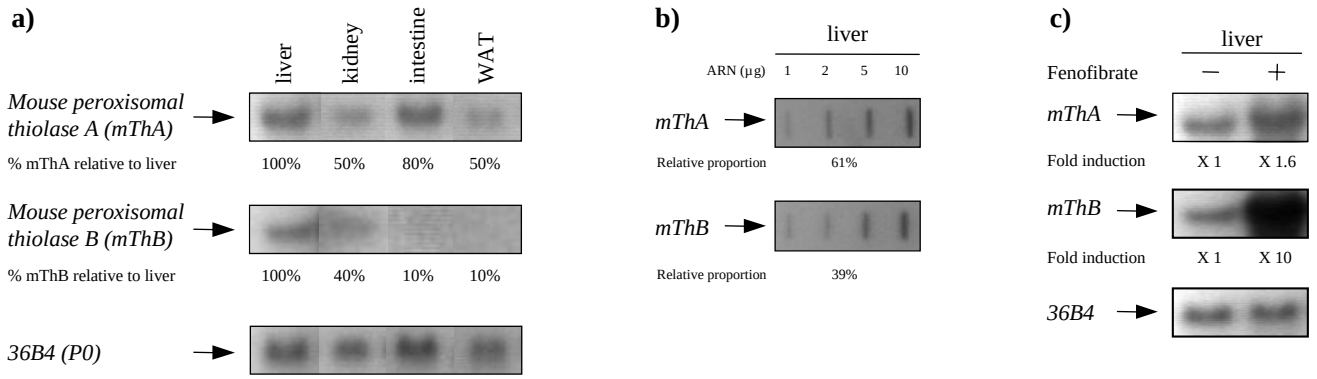

Figure 4

Mouse peroxisomal *3-ketoacyl-CoA thiolase A and B* mRNA levels in different tissues and effect of fenofibrate on their expression in liver. a) Total RNAs (15 µg per lane from 3 mice) extracted from different tissues were hybridized with ^{32}P-labelled cDNA probes: upper panel, mouse peroxisomal 3-ketoacyl-CoA thiolase A (mThA); middle panel, mouse peroxisomal 3-ketoacyl-CoA thiolase B (mThB); lower panel, 36B4 (acidic ribosomal phosphoprotein (P0)) as loading control. Peroxisomal *3-ketoacyl-CoA thiolase A* (*mThA*) or *thiolase B* (*mThB*) mRNA signal was quantified, standardized with *36B4* mRNA signal for the same tissue and expressed as a percentage in comparison with the signal observed in liver for each isoform. b) Relative proportions of *thiolase A* and *B* mRNAs in mouse liver by Slot blot analysis. 1, 2, 5 and 10 µg total RNAs extracted from mouse liver were deposed onto nitrocellulose membrane and then hybridized with ^{32}P-labelled cDNA probes: upper panel, mouse peroxisomal 3-ketoacyl-CoA thiolase A (mThA), and lower panel, mouse peroxisomal 3-ketoacyl-CoA thiolase B (mThB). The total signals of thiolase A and thiolase B were fixed to 100%. Relative proportions of *thiolase A* and *B* mRNAs were calculated and expressed as a percentage. c) Total RNAs (15 µg per lane from 3 independent animals for each condition) extracted from mouse liver of control and fenofibrate-treated mice were loaded. The membrane was hybridized with ^{32}P-labelled cDNA probes: upper panel, mouse peroxisomal 3-ketoacyl-CoA thiolase A (mThA); middle panel, mouse peroxisomal 3-ketoacyl-CoA thiolase B (mThB); lower panel, 36B4 (acidic ribosomal phosphoprotein (P0)) as loading control. Fold variation represents *mThA* (or *mThB*) mRNA / *36B4* mRNA variation. These values were fixed to 1 for liver of untreated mice.

At the protein level, thiolase sequences contain several well conserved characteristics between human, rat and mouse, both for thiolase A and B proteins. For example, the three thiolase signature patterns were found (framed in dotted lines on the sequences in figure 2): two of which were based on the regions around the cysteine residues (Cys123, Cys408) which are essential for the catalytic function and the third was based on a highly conserved region in the C-terminal part of these proteins containing histidine 377 also involved in the catalytic function [14]. The putative Peroxisomal Target Signal (PTS 2) was also identified in the N-terminal region of the protein. This signal is characteristic of peroxisomal thiolase and is cleaved after the import of the protein in peroxisomes [15], which results in a mature protein of 398 aa. At the 3' ends of the cDNAs, the stop codon and the putative polyadenylation signal AGTAAA were identified (figure 2). In this 3' region, downstream of the stop codon, the two *thiolase* cDNAs were very different (55% identity on a 136 nt stretch), which allowed us to prepare probes specific to each *thiolase* cDNA (underlined sequences, figure 2). These probes were used thereafter for Northern blot and Slot blot experiments (figure 4).

Nucleotide sequences of the 5'-flanking regions of the two *thiolase* genes are shown in figure 3. The 5'-flanking region of the *mThA* gene contains four putative Sp1 binding sites (GC boxes) at positions -30 to -21, -58 to -49, -105 to -93 and -306 to -297 and two inverted "CCAAT boxes" (i.e. "ATTGG sequences") at -107 to -102, and -122 to -117. However, there was no evidence for a typical TATA box sequence in the mouse *thiolase A* promoter region, as previously reported for numerous mammalian peroxisomal protein genes, such as the genes encoding rat *3-ketoacyl-CoA thiolase A* [8], human *3-ketoacyl-CoA thiolase* [11], rat *acyl-CoA oxidase* [16], rat *bifunctional enzyme* [17], human [18] and rat *catalase* [19]. In contrast, in the 5'-flanking region of the *mThB* gene, one TATA box-like AT rich sequence is present at -92 to -85 together with four GC boxes (at -26 to -16, -81 to -67, -120 to -114, and -271 to

-260) and two inverted "CCAAT boxes" (at -185 to -180 and -38 to -33). A similar putative sequence for the binding of NF-κB was present in both *mThA* and *mThB* genes, at position -170 to -159, and -292 to -283, respectively.

Tissue distribution of the two mouse peroxisomal thiolase transcripts and differential regulation by fenofibrate

Basal mRNA levels of mouse peroxisomal *3-ketoacyl-CoA thiolase A* and *B* in tissues with important lipid metabolism were determined by Northern blot analysis using specific probes for each *thiolase* mRNA (figure 4a). Peroxisomal *thiolase A* mRNA was mainly expressed in liver and intestine of mice. *Thiolase A* mRNA was less abundant in kidney and white adipose tissue. Peroxisomal *thiolase B* mRNA was mainly expressed in liver of mice and to a lesser extent in kidney, intestine and white adipose tissue. In the other tissues (skeletal muscle, heart and brain), *thiolase A* and *B* mRNAs were hardly detectable; their expression represented less than 10% of that found in liver (data not shown). The high expression of thiolase A and B mRNAs in the liver may be closely related to larger peroxisome volume in this organ, a major site of peroxisomal lipid oxidation. The smaller thiolase mRNA contents in kidney, adipose tissue, skeletal muscle and heart compared to liver were in concordance with what was observed in these same tissues for the mouse acyl CoA oxidase gene [20]. However, the thiolase mRNA level is low in brain whereas Nöhammer et al. [20] found considerable amount of acyl CoA oxidase mRNA.

The low thiolase mRNA level in the brain may appear as a paradox since in most human peroxisomal disorders, neurodevelopmental delay is a prominent abnormality. Even if the peroxisomal oxidation enzymes are lower expressed in brain compared to liver ([20], this work), their presence is essential for metabolism of specific lipids such as docosahexaenoic acid (C22:6n-3) and very long chain fatty acids. Moreover, Janssen et al. [21] described that neuronal migration alteration in Pex5 knock out mice could be significantly corrected by a partial reconstitution of peroxisomal function in liver suggesting that hepatic peroxisomal metabolism defect could affect the normal neuronal development. Taken together, these results suggest that peroxisomal disorders could not be due to a tissue-specific deficiency but rather to an association of multiple factors localized in several tissues.

In order to evaluate the relative proportions of *mThA* and *mThB* mRNA in liver, Slot blot experiments were carried out with mouse liver RNA (figure 4b). We found that hepatic *mThA* mRNA represented 60% of the overall hepatic *thiolase* mRNA population since *mThB* mRNA represents 40%. Comparable values were obtained in kidney, intestine and white adipose tissue (data not shown). The results obtained in mouse liver were different from those obtained in rat liver since in the absence of treatment with a peroxisome proliferator, only *thiolase A* mRNA was detected [8,9]. After peroxisome proliferator treatment, rThB gene expression was greatly induced whereas rThA gene expression was increased less than two-fold [8]. In order to evaluate if this differential gene regulation by peroxisome proliferators occurs also in mouse, we compared the ratio of the mThA and the mThB mRNAs between mice treated or not with fenofibrate, a peroxisome proliferator. As shown in figure 4c, mThB mRNA level was increased more than ten-fold whereas mThA mRNA level was increased two-fold. These results are in agreement with what is described in the rat.

Thiolase enzyme activity assays

We measured the enzymatic activity of the peroxisomal 3-ketoacyl-CoA thiolase by using an immunodepletion-based method. Because of the presence of distinct thiolases both in mitochondria and peroxisomes, we used the indirect method of Miyazawa et al. [6] to specifically determine peroxisomal 3-ketoacyl-CoA thiolase A+B activities. It consists in the measurement of thiolase activities before and after an immunodepletion with an antibody directed against both thiolase A and thiolase B proteins. Since no substrate specificity has been described to distinguish between rat thiolase A and B activities [22,23], differentiating their activities in the mouse was not feasible. Therefore, thiolase activities were measured by using 3-keto-octanoyl-CoA as substrate, as previously described by Miyazawa et al. [6].

To verify the complete immunodepletion of peroxisomal 3-ketoacyl-CoA thiolase from the homogenate, samples of the homogenate before and after immunodepletion were separated on a polyacrylamide gel and analysed by Western blotting. Membranes were incubated with an anti-peroxisomal 3-ketoacyl-CoA thiolase antibody (figure 5a, lane a) and after stripping, membranes were reprobed with an anti-mitochondrial 3-ketoacyl-CoA thiolase antibody (T1) to verify the specificity of the immunodepletion (figure 5a, lane b). Peroxisomal thiolase A+B proteins were totally absent from the homogenate after immunodepletion with 5 μl and 10 μl of anti-PTL antibody (figure 5a, lane a). Under these conditions, the amount of mitochondrial 3-ketoacyl-CoA thiolase was not affected by the immunodepletion showing the specificity of this technique (figure 5a, lane b). Once these verifications were done, the peroxisomal thiolase activity was measured in the four tissues showing the strongest *thiolase* mRNA levels (figure 5b). Thiolase A+B activities were detected in liver, kidney, intestine and white adipose tissue. No significant differences were observed between liver and the other three tissues. Discrepancies between enzymatic activity and mRNA level have already been

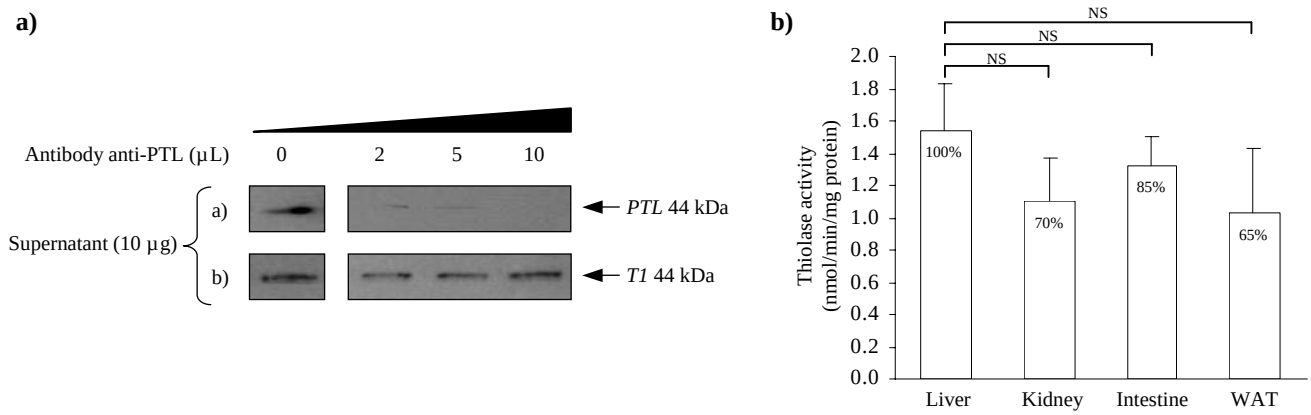

Figure 5

Tissue distribution of mouse peroxisomal *3-ketoacyl-CoA thiolase* A+B activities. a) Sample immunodepletion with antibody anti-peroxisomal 3-ketoacyl-CoA thiolase A and B proteins (anti-PTL). The samples of homogenate before and after immunodepletion with the indicated amount of the PTL antibody were subjected to electrophoresis by SDS/PAGE and incubated after Western blot with the anti-PTL antibody (lane a) or with the anti-T1 antibody directed against the mitochondrial thiolase (lane b). The approximate size of the two proteins (in kDa) is indicated on the right. b) Total peroxisomal 3-ketoacyl-CoA thiolase A+B activities were measured in different tissues by subtracting activities determined on homogenate after immunodepletion from activities determined on homogenate before immunodepletion. Thiolase activities in different tissues are indicated in percentages in the bar chart and values were fixed to 100% for the liver. Means ± S.E.M of thiolase activities correspond to three independent experiments performed on three mice. No significant difference (NS) between liver and the other three tissues was found with the Student's *t* test (P > 0.05).

described for some enzymes, particularly under treatment conditions [24]. Moreover, under our conditions, thiolase activities resulted from measurement of both thiolase A and B activities. As described in the rat, the two enzymes could have different stability [22] or the two thiolase mRNAs could be differently translated. Thus, differences observed between thiolase A and B protein and mRNA levels may more likely reflect the inability to discriminate between thiolase A and thiolase B activities.

Conclusions

The *thiolase A* and *B* genes are approximately 9 kb in length, and include 12 exons and 11 introns. The corresponding *thiolase A* and *B* cDNAs possess an open reading frame of 1272 nucleotides encoding a precursor protein of 424 amino acids. *Thiolase A* and *B* transcripts are mostly expressed in the liver of mice and differently regulated by fenofibrate (2-fold *vs* 10-fold). At the enzymatic level, thiolase A+B activities are detected in liver, kidney, intestine and white adipose tissue.

Methods

Isolation of genomic clones and DNA sequence analysis

A 129SV mouse genomic library (Clontech) was screened using ^{32}P-labelled common exon 9 rat thiolase probe. Positive clones were isolated and purified by three successive rounds of screening. Two distinct genomic clones were obtained and sequenced (Genome Express S.A., Grenoble, France); they corresponded to two different mouse *thiolase* genes. The putative consensus sequences for eukaryotic transcription factors in the promoter regions were identified by using TRANSFAC V4.0 software http://www.cbil.upenn.edu/cgi-bin/tess/tess.

Southern blot analysis

Mouse genomic DNA (15 µg) was digested at 37°C for 4 h with different enzymes: *Pst* I, *Hin*dIII, *Bam*HI and *Eco*RI (figure 1a). Genomic DNA was subjected to 0.8% agarose gel electrophoresis and blotted to Hybond N filters (Amersham). Membrane was hybridized with the same thiolase probe used for genomic library screening (see above). cDNA probe was labelled by random priming using [α-^{32}P]dCTP (Amersham) and purified by centrifugation through Pharmacia ProbeQuant G50 microcolumns. Hybridization was performed according to Church

and Gilbert [25]. Blots were washed at high stringency (65°C, 0.5 × SSC, 0.1% SDS) before autoradiography using X-ray films (Amersham).

Determination of transcription initiation sites by ribonuclease protection assay

Two probes were constructed by PCR using mouse genomic DNA. The thiolase A probe (named +1mThA probe and extending from -188 to +388 in the promoter region of mouse thiolase A (figure 3a)) was PCR-amplified with primers 5'-AAAAGGTTGTGCAAACTTGGAAG-3' and 5'-AAGCCTCCACGGCTGGCGCGGCC-3'. The thiolase B probe (named +1mThB probe and extending from -152 to +266 in the promoter region of mouse thiolase B (figure 3b)) was PCR-amplified with primers 5'-CCATCACAGGTGTACATGGAC-3' and 5'-AAGCAGCCACGGCTGGCGCGGCC-3'. The two fragments were subcloned into pGEM®-T Easy vector (Promega) and verified by sequencing. These two vectors were linearized with *Sal*I and used to synthesize the RNA probes by *in vitro* transcription (Riboprobe® in vitro transcription systems, Promega) using [α-^{32}P]UTP (800 Ci/mmol, Amersham) and T7 RNA polymerase. The radiolabelled probes were gel-purified and hybridized to 15 μg (for +1mThA probe) or 10 μg (for +1mThB probe) of fenofibrate-treated mouse liver RNA in a hybridization buffer (80% formamide, 10 mM PIPES, pH6.8, 0.25 mM EDTA, 0.1 M NaCl) overnight at 45°C. The reaction mixtures were digested with 2 μg of ribonuclease A (Sigma) and 36 Units of ribonuclease T1 (Sigma) for 30 min at 37°C. Protected fragments were visualized on a denaturing 7 M urea/5% polyacrylamide gel. Plasmids containing the +1mThA and +1mThB probes were sequenced with primers 5'-AAAAGGTTGTGCAAACTTGGAAG-3', 5'-CCATCACAGGTGTACATGGAC-3' respectively, and run in parallel.

3' rapid amplification of the thiolase A and B cDNA ends (3' RACE)

Three prime ends of the *thiolase A* and *B* cDNAs were prepared with the Marathon™ cDNA amplification kit (Clontech) using 3 μg of total liver RNA extracted from mice. 3' RACE reaction was performed using gene-specific primer (GSP) deduced from partial cDNAs of mouse *thiolase A* and *thiolase B* (GenBank accession nos BC012400 and BC019882, respectively). A GSP common thiolase reverse primer (5'-ACTCCAGTCAGGTGAGTGATG-3' (21-mer, located in exon 9)) and a forward primer corresponding to the Marathon™ adapter: (5'-CCATCCTAATACGACTCACTATAGGGC-3' (27-mer)) were used. Briefly, total RNA was reverse-transcribed with Moloney murine leukemia virus-reverse transcriptase using poly(dT)$_{30}$-NN as primer. Second strand synthesis was performed with a mixture of *E. coli* DNA polymerase I, Rnase H, and DNA ligase (Promega). Following the creation of blunt ends with T4 DNA polymerase, cDNAs were ligated with the Marathon cDNA-adapter. Then, the 3' cDNA ends were amplified using this cDNA population with the Expand Long Template PCR System (Roche) for 30 cycles using the following steps: 95°C for 30 s, 56°C for 30 s and 68°C for 1 min 30. The PCR products were analyzed by electrophoresis on 1.2% agarose gel in TAE buffer. From each thiolase A and B 3' RACE, a single amplification product was obtained with a size of 717 bp and 750 bp, respectively. These PCR products were gel-purified, cloned in pGEM®-T Easy vector (Promega) and sequenced (Genome Express S.A., Grenoble, France).

Cloning of thiolase A and B cDNAs

Thiolase cDNAs were amplified by RT-PCR with 3 μg of total liver RNA extracted from mice. The *thiolase A* cDNA was amplified using a specific sense primer 5'-GACGTAGAAGGCTGTCCTGCGCCTGCGCCC-3' (280 to 309: position on the mouse *thiolase A* gene sequence (GenBank accession no AY304542)) and an antisense primer 5'-GAGACAGTACACATTTACTGCATCCCTCCC-3' (9152 to 9181: position on the mouse *thiolase A* gene sequence (GenBank accession no AY304542)). For the *thiolase B* cDNA, the primers used were 5'-TTCACTGGGGTGACGCCAGAGCGCT-3' (46 to 70: position on mRNA relative to +1 initiation transcription site deduced from the mouse *thiolase B* gene sequence (GenBank accession no AC055818)) and 5'-CAGTTCACCCACGTTTACTGTGCCCATCAC-3' (1637 to 1666: position on mRNA relative to +1 initiation transcription site deduced from the mouse *thiolase B* gene sequence (GenBank accession no AC055818)). The two *thiolase* cDNA fragments were cloned in pGEM®-T Easy vector (Promega), completely sequenced (Genome Express S.A., Grenoble, France) and deposited in the Genbank database (accession nos: AY273811 for *mThA* cDNA and AY273812 for *mThB* cDNA).

Animals and fenofibrate treatment

C57BL/6J male mice (aged 6 months, n = 8) were purchased from IFFA CREDO (L'Arbresle, France). Mice (n = 4) were kept at 22°C with equal periods of darkness and light and had free access to water and a standard chow containing 4.3% (w/v) lipid in total calories (U.A.R. A-03, Epinay sur Orge, France). Mice were treated for 14 days by gavage (200 μl/day) with fenofibrate (100 mg. kg^{-1}. day^{-1}, from Sigma) dispersed into water containing 3% arabic gum (Merck). Treatments were performed between 8.00 a.m. and 10.00 a.m.. Animals were sacrificed by cervical dislocation and the following tissues were rapidly excised: liver, kidney, heart, whole brain, skeletal muscle, enterocytes (intestinal mucous membrane) and white adipose tissue (peritoneal, retroperitoneal and epididymal regions). Tissues were snap-frozen in liquid nitrogen and stored at -80°C until RNA and protein extractions. The

general guideline for the care and use of laboratory animals was followed.

RNA analysis

Northern blotting analysis

For the isolation of total RNA, each frozen tissue (20–200 mg) was homogenized with an Ultra-Turrax homogenizer (IKA Labortechnik) in 3 ml of a mixture containing guanidium thiocyanate and extracted according to Chomczynski and Sacchi [26]. Total RNA from 3 independent animals was denatured, subjected to electrophoresis on a 1% (w/v) agarose gel containing 6% (v/v) formaldehyde and transferred overnight onto Hybond N filters (Amersham). Probes labelling and hybridization conditions were the same as those described for Southern blot analysis (see above). Specific mouse peroxisomal thiolase A and B probes (underlined sequences, figure 2) were used for hybridization. To monitor gel loading, a 443 bp mouse acidic ribosomal phosphoprotein-36B4 (P0) probe was generated by RT-PCR from mouse liver RNA using primers based on the published cDNA sequence of the mouse *acidic ribosomal phosphoprotein* gene (GenBank accession no X15267) [27]. The forward and reverse PCR primers were respectively: 5'-AACGTGGGCTCCAAGCAGATG-3' (position on mRNA: 173 to 193) and: 5'-GAGATGTTCAGCATGTTCAGCAG-3' (position on mRNA: 593 to 615). Band intensities were determined by densitometry with an Alpha-Imager™ 1220 (Alpha Innotech Corporation).

Slot blot analysis

Increasing quantities of total RNA (1, 2, 5 and 10 μg) were denatured and deposed onto Hybond N filters in a Slot blot equipment (Hoefer scientific instruments). Experiments were performed in 20 × SSC under vacuum and washed twice in 10 × SSC for 5 min. Then, membranes were wetted at room temperature for 10 min and hybridization with thiolase probes was performed as Northern blot experiments. Linearity of the relationship between signal intensity and RNA concentration was confirmed for each sample. Band intensities (for 2 μg RNA) were determined by densitometry with an Alpha-Imager™ 1220 (Alpha Innotech Corporation).

Protein analysis

SDS-PAGE and immunoblotting

Protein extraction

Liver samples were homogenized with an Ultra-Turrax homogenizer (IKA Labortechnik) and then with a Teflon pestle (Bioblock) in a lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P40, 0.5% (w/v) sodium deoxycholate, 2 mM EDTA, 50 mM NaF) in the presence of a mixture of protease inhibitors (Roche). Homogenates were briefly centrifuged to remove the debris. Total protein concentration was determined by the Bradford technique with the Bio-Rad reagent (Biorad) and with bovine serum albumin as a standard.

Thiolase immunodepletion and enzymatic activity determination

The rabbit polyclonal antibodies directed against both thiolase A and B proteins (named PTL) and against mitochondrial thiolase (named T1) were a gift from Dr. T. Hashimoto and Dr. N. Usuda (Shinshu University School of Medecine, Japan) and have been previously described [6].

Homogenates of proteins (25 μg) were incubated at room temperature for 1 h with 5 μl of anti-peroxisomal 3-ketoacyl-CoA thiolase antibody (PTL) in a buffer containing 25 mM Tris-HCl pH 8.0, 0.5% (v/v) Nonidet P40, 0.25% (w/v) sodium deoxycholate, 1 mM EDTA, 25 mM NaF. Six μg of protein A-Sepharose CL-4B (Sigma Chemical Co., St Louis, MO) in suspension in the lysis buffer were added to the mixture and were incubated for 30 min at room temperature to allow the formation of the complex [antibody-antigen-protein A-Sepharose]. This complex was sedimented by centrifugation at 10 000 g for 2 min.

For immunoblotting, 10 μg of homogenate (with or without immunodepletion) were separated on 10% (w/v) polyacrylamide gels in the presence of 0.1% (w/v) SDS and transferred onto nitrocellulose membranes (Bio-Rad). After membrane saturation at room temperature for 1 h 30 with TBS (0.1 M Tris-HCl pH8.0, 0.15 M NaCl) containing 0.05% (v/v) Tween 20 and 1% (w/v) BSA, blots were incubated in the same solution for 1 h with rabbit polyclonal antibodies directed againt thiolase A and thiolase B proteins (PTL, diluted to 1:15000) or directed against mitochondrial 3-ketoacyl-CoA thiolase (T1, diluted to 1:30000). After three washes in TBS containing 0.05% (v/v) Tween 20, primary antibody was detected using peroxidase-conjugated anti-rabbit (1:30000). The bands labelled with the antibodies were visualized using a Western blotting Chemiluminescence luminol reagent (Santa Cruz Biotechnology) by exposure to X-ray films (Amersham). Membranes were stripped for 15 min at room temperature in a Restore buffer (Pierce, Montluçon, France) and four washes in TBS were performed before new hybridization.

Enzyme activity assays

The 3-ketoacyl-CoA thiolase activities were determined at 25°C by measuring the absorbance decrease at 303 nm due to Mg^{2+}-enolate cleavage [28]. The reaction mixture (500 μl) contained: 5 μg of protein homogenate (with or without immunodepletion), 100 mM Tris-HCl pH8.0, 25 mM $MgCl_2$ and 10 μM 3-keto-octanoyl-CoA (a generous gift of Dr. T. Hashimoto and Dr. N. Usuda, Shinshu University School of Medecine, Japan). The enzymatic reaction was activated by adding Coenzyme A at a final

concentration of 100 μM (Sigma Chemical Co., St Louis, MO). Under these assay conditions, the molar absorption coefficient was determined by the method of Staack et al. [29]: 3-keto-octanoyl-CoA, 14400 $M.cm^{-1}$. Thiolase A+B activities were measured on homogenates before and after immunodepletion. Peroxisomal 3-ketoacyl-CoA thiolase A+B activity was calculated as the difference between these two determinations. Units of thiolase activities are expressed as nmol of substrate utilized per mg of total protein per min. The results are expressed as means ± S.E.M. The significance of differences between tissues was determined by Student's *t* test (NS > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001).

List of abbreviations used

aa, amino acid(s); AOX, peroxisomal fatty acyl-CoA oxidase; D-PBE, peroxisomal D-3-hydroxyacyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme; GSP, Gene Specific Primer; L-PBE, L-3-hydroxyacyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme; mThA (or mThB), mouse peroxisomal 3-ketoacyl-CoA thiolase A (or B); NFκB, nuclear factor kappa B; PTL, antibody against peroxisomal 3-ketoacyl-CoA thiolase A and B proteins; PTS, Peroxisomal Target Signal; 3' RACE, 3' rapid amplification of cDNA ends; rThA (or rThB), rat peroxisomal 3-ketoacyl-CoA thiolase A (or B); SCPx, sterol carrier protein x; T1, antibody against mitochondrial short chain-specific 3-ketoacyl-CoA thiolase; WAT, white adipose tissue; 36B4, acidic ribosomal phosphoprotein (P0).

Authors' contributions

GC carried out the transcription initiation sites determination, the Northern blot experiments and the enzymatic activity assays. MCC performed gene cloning and the sequence analysis. PE cloned the cDNAs and performed the Slot blot analysis. PM, TP and GC conducted the experiments on animals. VNF participated to the cloning of genes. GC and VNF wrote the draft of the manuscript. NL and VNF conceived the study and supervised the work. All authors read and approved the final manuscript.

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