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Original article

Cloning, chromosome mapping and expression pattern of porcine *PLIN* and *M6PRBP1* genes

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Abstract – The PAT proteins, named after the three PLIN/ADRP/TIP47 (PAT) proteins, PLIN for perilipin, ADRP for adipose differentiation-related protein and TIP47 for tail-interacting protein of 47 kDa, now officially named M6PRBP1 for mannose-6-phosphate receptor binding protein 1, is a set of intracellular lipid droplet binding proteins. They are localized in the outer membrane monolayer enveloping lipid droplets and are involved in the metabolism of intracellular lipid. This work describes the cloning and sequencing of porcine *PLIN* and *M6PRBP1* cDNAs, the chromosome mapping of these two genes, as well as the expression pattern of porcine PAT genes. Sequence analysis shows that the porcine *PLIN* cDNA contains an open reading frame of 1551 bp encoding 516 amino acids and that the porcine *M6PRBP1* cDNA contains a coding region of 1320 bp encoding 439 amino acids. Comparison of PLIN and M6PRBP1 amino-acid sequences among various species reveals that porcine and bovine proteins are the most conserved. Porcine *PLIN* and *M6PRBP1* genes have been mapped to pig chromosomes 7 and 2, respectively, by radiation hybrid analysis using the IMpRH panel. Expression analyses in pig showed a high expression of *PLIN* mRNA in adipose tissue, *M6PRBP1* mRNA in small intestine, kidney and spleen and *ADRP* mRNA in adipose tissue, lung and spleen.

pig / *PLIN* / *M6PRBP1* / cDNA cloning / chromosome mapping / tissue expression pattern

1. INTRODUCTION

Intracellular neutral lipid storage droplets, essential organelles of eukaryotic cells, are required for energy balance, membrane biosynthesis, cholesterol metabolism and lipid trafficking. Animal lipid storage droplets contain

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a set of proteins called the PAT domain family proteins that include perilipin (PLIN), adipose differentiation-related protein (ADRP), and mannose-6-phosphate receptor binding protein 1 (M6PRBP1) previously named tail-interacting protein of 47 kDa (TIP47). The PAT family proteins share extensive amino acid sequence similarity, especially at their N-termini. They are associated with lipid droplets, and involved in the lipid droplets formation and degradation [9,18]. Perilipin is a phosphoprotein involved in the hormone-stimulated lipolysis and its expression is highly restricted to adipocytes and steroidogenic cells [5, 20]. Under hormone stimulation, perilipin is phosphorylated by cAMP-dependent protein kinase A (PKA) and recruits hormone-sensitive lipase (HSL) to the lipid droplets, thereby promoting lipolysis [19, 21]. PLIN knockout mice have a reduced basal adipocyte lipolysis, are lean and resistant to diet-induced obesity [16, 24]. Furthermore, it has been shown that in human adipose tissue, perilipin expression increases with obesity [11], that perilipin promotes triacylglycerol (TAG) storage in adipocytes by regulating the rate of basal lipolysis and also, that it is required to maximize hormonally stimulated lipolysis [13, 25]. M6PRBP1 was first identified as a binding partner of the mannose 6-phosphate receptor (MPR) required for its transport from endosomes to the trans-Golgi network [4]. Recent research has indicated that M6PRBP1 is a key effector for Rab9 localization [1]. M6PRBP1 is known to associate with lipid droplets and to share a high level of sequence similarity with ADRP. Unlike PLIN and ADRP, which associate exclusively with lipid droplets, M6PRBP1 exists in both droplet-associated and soluble forms [18,27]. The functions of M6PRBP1 in lipid metabolism are not as well known as those of PLIN.

The aim of this work was to sequence the porcine *PLIN* and *M6PRBP1* cDNAs, to map the two genes, and to analyze the tissue-specific expression patterns of the PAT family mRNAs. It represents an initial step toward the detailed biochemical and functional analyses of PAT proteins in pig.

2. MATERIALS AND METHODS

2.1. Animals

Meishan pigs (25–30 kg) were obtained from the Animal Center of Huazhong Agricultural University (Wuhan, China). The Hubei Province Committee on Laboratory Animal Care approved all experimental procedures and housing. After slaughter, white adipose tissue, liver, kidney, heart, muscle, lung, spleen, pancreas, small intestine, brain and stomach were quickly

dissected and frozen in liquid nitrogen and then stored at –70 °C until extraction for total RNA. Three pigs were used for the expression analyses.

2.2. Total RNA isolation and reverse transcription

Total RNA was extracted and purified from the frozen tissue with Trizol Reagent (Sangon, Shanghai) according to the manufacturer's protocol, and subsequently treated with DnaseI (Takara, Dalian, RNase-free) to degrade possible genomic DNA. Total RNA concentrations were calculated from the optical density (OD) value at wavelength 260 nm and the ratios OD260/OD280 were determined. The quality of the preparations was also checked by denaturing agarose gel electrophoresis. Total RNA was used as template for oligo (dT)18 primed reverse transcription using 200 U of M-MLV reverse transcriptase (Promega), 0.5 mM of each dNTP and 3 mM $MgCl_2$.

2.3. Cloning of pig *PLIN* and *M6PRBP1* cDNAs

All primers used in this work are included in Table I. Primers P1 and P2 designed from the consensus sequence between human and mouse *PLIN* cDNAs (GenBank, NM_002666 and NM_175640), were used to amplify the central region of porcine *PLIN* cDNA, while primers (P3, P4) and (P5, P6) were used to amplify the 5' and the 3'-end coding sequences, respectively. The complete porcine *PLIN* cDNA sequence was obtained by assembling these three fragments.

Similarly, primers P12 and P13, designed from the consensus sequence between human and mouse *M6PRBP1* cDNAs (GenBank, AF057140 and AK004970), were used to amplify the central region of porcine *M6PRBP1* cDNA and primers (P14, P15) and (P16, P17) were used to amplify the 5' and the 3'-end coding sequences, respectively.

2.4. Radiation hybrid mapping

PLIN and *M6PRBP1* genes were mapped by radiation hybrid analysis, which was carried out using DNA samples isolated from the hybrid clones included in the INRA-University of Minnesota porcine Radiation Hybrid (IMpRH) panel [10, 28]. The presence or absence of the porcine genes in each of the DNA samples was determined by PCR amplification of a fragment of each gene. For *PLIN*, primers P9 and P10 (Tab. I) were designed from the sequence of intron 6 amplified by primers P7 and P8. PCR consisted in an initial

Table I. Primer sequences used in this study.

Primer	Sequence (5'→ 3')		Use	Origin of sequence
P1	AGCACTAAGGAAGCCCACCCC	forward	amplify the central region of pig *PLIN* cDNA	human-mouse consensus sequence
P2	CGGAATTCGCTCTCGGGC	reverse	amplify the central region of pig *PLIN* cDNA	human-mouse consensus sequence
P3	TATGAACATTAAAGGGAAGAAG	forward	amplify the 5'-end coding sequence of pig *PLIN* cDNA	human
P4	CGGAGGCGGGTGGAGATTGT	reverse	amplify the 5'-end coding sequence of pig *PLIN* cDNA	the sequence from the amplified products of primer P1 and P2
P5	TCACGATGACCAGACGGACACAG	forward	amplify the 3'-end coding sequence of pig *PLIN* cDNA	the sequence from the amplified products of primer P1 and P2
P6	CGGGGCGCGGCGGCTGGT	reverse	amplify the 3'-end coding sequence of pig *PLIN* cDNA	human
P7	GGAGGATCACGATGACCAGACG	forward	perilipin intron 6 cloning	pig
P8	CACCGCTTTGCCCAGGAA	reverse	perilipin intron 6 cloning	pig
P9	TCACGATGACCAGACGGACACAG	forward	perilipin mapping, perilipin semi-quantitative	pig
P10	CTCTTGGGTAGGTCGCTTCTGCTT	reverse	perilipin mapping	pig
P11	GCCCAAGTCACGAGGGAGAT	reverse	perilipin semi-quantitative	pig
P12	GCTCAGCCGATCCTCTCCAAG	forward	amplify the central region of *M6PRBP1* cDNA	human-mouse consensus sequence pig
P13	CAGCTGAGCCACATCTGGTG	reverse	amplify the central region of pig *M6PRBP1* cDNA	human-mouse consensus sequence
P14	CTTCCAAGCTGGTCTTGAA	forward	amplify the 5'-end coding sequence of pig *M6PRBP1* cDNA	human
P15	TCCACCCACTCCTCCGACTT	reverse	amplify the 5'-end coding pig *M6PRBP1* cDNA	the sequence from the amplified products of primer P12 and P13
P16	CAGAGCGGCGTGGACCTGA	forward	amplify the 3'-end coding sequence of pig *M6PRBP1* cDNA	the sequence from the amplified products of primer P12 and P13
P17	GCCTTAGCTTCCCAAGTGGA	reverse	amplify the 3'-end coding sequence of pig *M6PRBP1* cDNA	human
P18	GGCCGGAATGCCACTCATCA	forward	*M6PRBP1* mapping	pig
P19	TCCCCTGTGGGCGTATTCACTG	reverse	*M6PRBP1* mapping	pig
P20	GGCAAGTCGGAGGAGTGGGT	forward	*M6PRBP1* semi-quantitative	pig
P21	CAGGCTGAGTGCTTGCGACA	reverse	*M6PRBP1* semi-quantitative	pig
P22	CCTGGGAAGTCGGATGATGC	forward	*ADRP* semi-quantitative	pig
P23	TGGTAACCCTCGGATGTTGGA	reverse	*ADRP* semi-quantitative	pig
P24	GGTCATCACCATCGGCAACG	forward	β-actin, internal control	pig
P25	TGGAAGGTGGACAGCGAGGC	reverse	β-actin, internal control	pig

step at 94 °C for 3 min and 35 cycles at 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s. For *M6PRBP1*, primers P18 and P19 (Tab. I) and the following PCR conditions were used: an initial denaturation step at 94 °C for 3 min and 35 cycles at 94 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s. The DNA of each clone in the RH panel was amplified twice in order to confirm results. The results were analyzed using the IMpRH mapping tool (http://www.imprh.toulouse.inra.fr) developed by [17]. Chromosomal regional localizations were deduced either from the position of the closest linked marker located on the cytogenetic map for *PLIN* or from the interval on the cytogenetic map of the linkage group carrying the closest linked marker for *M6PRBP1*.

2.5. Semi-quantitative RT-PCR

Total RNA samples prepared from eleven different tissues from Meishan pigs were reverse transcribed by a standard procedure (Promega) using M-MLV reverse transcriptase and an oligo(dT) 18 primer. The reverse transcription reaction products were used as templates in PCR amplifications carried out with the following pig-specific primer pairs, P9 and P11 for *PLIN*, P22 and P23 for *ADRP* (designed from pig *ADRP*, AY550037), P20 and P21 for *M6PRBP1* and P24 and P25 for β-actin (designed from pig β-actin, SSU07786). To avoid the PCR entering plateau stages, the number of cycles was adapted in each case. To assess relative mRNA levels of *PLIN*, *ADRP* and *M6PRBP1* in porcine tissues, the pig housekeeping gene β-actin was used as the internal standard. We performed controls with different dilutions of cDNA template to ascertain the linearity of the amplification (data not shown). Quantitative analyses of relative mRNAs levels were carried out with the Quantity One V 4.313 image analyzing system. The experiment was carried out on three pigs ($n = 3$) and each value was expressed as the mean value ± SD.

3. RESULTS AND DISCUSSION

3.1. Cloning of porcine *PLIN* and *M6PRBP1* cDNAs

Using adipose tissue and small intestine total RNA, we cloned the porcine *PLIN* and *M6PRBP1* cDNAs, respectively. Porcine *PLIN* cDNA (GeneBank, AY973170) has an ORF of 1551 nucleotides, encoding a 516 amino-acid peptide and porcine *M6PRBP1* cDNA (GeneBank, AY939831) contains a 1320 bp coding region encoding a 439 amino-acid protein. Table II shows the percentages of sequence similarity of porcine PLIN and M6PRBP1 protein sequences

with those of cattle, dog, man, mouse, rat, monkey, chicken and frog. The highest sequence similarity is observed with the bovine proteins.

The porcine PLIN protein contains six consensus sites for phosphorylation by cAMP-dependent protein kinase A (PKA), serines 81, 277, 436, 491, 516, and threonine 431 (Tab. III). It has been shown that phosphorylation of PLIN is a key process in lipolysis [3, 26] and that the three carboxyl-terminal sites (Ser 433, Ser 492, Ser 517) are critical to protect the lipid droplets from lipases [26]. Furthermore, protein kinase A-mediated phosphorylation of PLIN serine 492 promotes the fragmentation and dispersion of lipid droplets [15]. Pig and mouse PLIN each have a unique phosphorylation site *i.e.* threonine 431 and serine 222, respectively.

A previous study has indicated that the mouse *PLIN* gene has four mRNA variants that yield four proteins, perilipin A (516 amino-acids), B (421 amino-acids), C (347 amino-acids), and D (244 amino-acids), with the same N-termini sequence and distinct C-termini sequences [14]. The human *PLIN* gene has two mRNA variants that yield two proteins, perilipin A and B. Perilipin A is the predominant protein isoform and plays key roles in facilitating both the storage and hydrolysis of triacylglycerol (TGA) [7]. Based on the different splicing sites present in the mouse and human genes, we designed different primers to try to amplify porcine *PLIN* mRNA variants in different tissues, but failed to find other variants.

Porcine PLIN, ADRP and M6PRBP1 proteins share a typical PAT-1 domain, a 33-mer motif, and a PAT-C domain (Tab. IV). The PAT-1 domain is a highly conserved region at the N-terminus of the PAT family proteins [13]. The PAT-1 domain of ADRP protein shares 60.9% and 40.2% sequence similarity with that of M6PRBP1 and PLIN proteins (Tab. IV). Sequence analyses based upon neighbor-joining comparisons confirm that PLIN, ADRP, and M6PRBP1 proteins form separate groups (Fig. 1).

3.2. Chromosomal mapping of porcine *PLIN* and *M6PRBP1* genes

Porcine *PLIN* and *M6PRBP1* genes were mapped by radiation hybrid analysis using the 118 clones of the IMpRH panel. PCR results were submitted to the INRA-Minnesota Porcine Radiation Hybrid (IMpRH) Server and retention frequencies were calculated. *PLIN* and *M6PRBP1* genes showed a retention frequency of 13% and 11%, respectively. Thus, the porcine *PLIN* gene is mapped to SSC7q at a distance of 68 cR from the most significantly linked marker *SWR1210* (LOD score threshold 5.21). Since *SWR1210* has been previously localized by FISH on SSC7q [22], we could deduce

Table II. Sequence similarity percentages of porcine PLIN and M6PRBP1 at the nucleotide and amino acid levels in different species.

	Cattle (%)	**Dog** (%)	**Human** (%)	**Mouse** (%)	**Rat** (%)	**Monkey** (%)	**Chicken** (%)	**Frog** (%)
***PLIN* coding sequence**	87.7	85.5	85.0	80.6	79.6	78.2	55.8	–
PLIN protein	86.0	82.4	82.4	79.7	77.8	75.2	36.3	–
***M6PRBP1* coding sequence**	89.4	72.0	83.5	75.9	74.8	–	–	55.7
M6PRBP1 protein	89.7	69.8	80.9	73.1	72.9	–	–	50.3

Table III. cAMP-dependent protein kinase phosphorylation sites in the perilipin protein.

	1	2	3	4	5	6	7
Mouse	78–81	219–222	273–276	—	430–433	489–492	514–517
	RRLS	*RRVS*	**RR*Q*S**		**R*KG*S**	**RRVS**	**RKKS**
Pig	78–81	—	274–277	428–431	433–436	488–491	513–516
	RRLS		**RR*Q*S**	*RRET*	**R*RG*S**	**RRVS**	**RKKS**
Cattle	78–81	—	274–277	—	433–436	488–491	513–516
	RRLS		**RR*R*S**		**R*RG*S**	**RRVS**	**RKKS**
Human	78–81	—	274–277	—	433–436	494–497	519–522
	RRLS		**RR*R*S**		**R*RA*S**	**RRVS**	**RKKS**

cAMP-dependent protein kinase phosphorylation sites (consensus pattern: [RK] (2)-X-[ST]) were predicated using Prosite (http://www.expasy.org/prosite/). S is the serine phosphorylation site, T is the threonine phosphorylation site.

Table IV. PAT-1 domain, 33-mer motif and PAT-C domain in porcine PAT family proteins. The PAT-1 domain is a highly conserved region at the N-terminal end and the 33-mer motif and PAT-C domain are conserved regions at the C-terminal end. Values indicate the percentage of amino-acid sequence similarity as compared with ADRP.

	Full length (a.a.)	Identity with ADRP	PAT-1 domain (a.a.)	Identity with ADRP	33-mer motif (a.a.)	Identity with ADRP	PAT-C domain (a.a.)	Identity with ADRP
ADRP	459		9–100		125–157		175–401	
M6PRBP1	439	37.3%	23–114	60.9%	143–175	51.5%	193–422	36.9%
PLIN	516	19.2%	17–108	40.2%	126–158	18.2%	176–407	17.2%

the following cytogenetic localization *i.e.* SSC7q11-q14 for *PLIN*. The gene *M6PRBP1*was mapped to SSC2 at a distance of 29 cR from the most significantly linked marker *SW1655* (LOD score threshold 9.15). Marker *SW1655* has not been localized by FISH but it has been shown to be situated between the marker *S*0170 and the gene *ADM* on the porcine linkage map (Arkdb

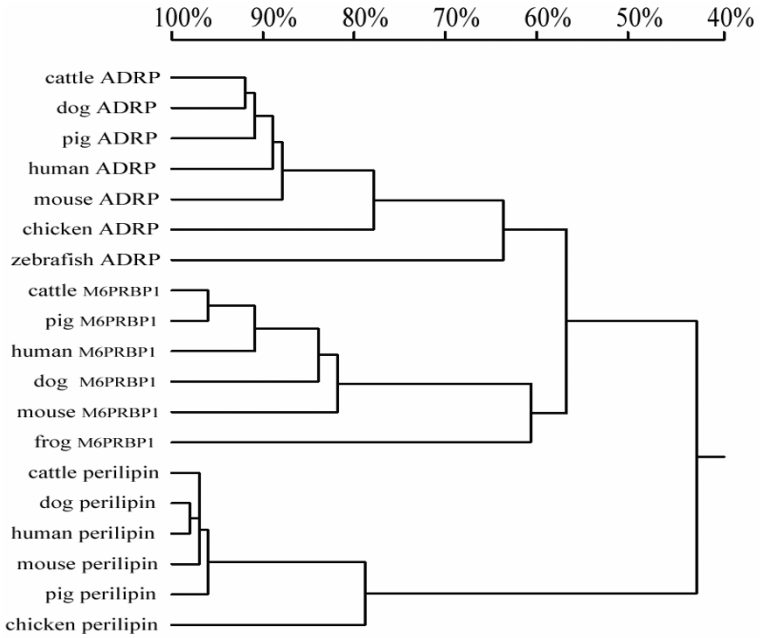

Figure 1. Phylogenetic tree of the PAT-1 domains in PAT family proteins. PAT proteins were identified with NCBI. The phylogenetic tree was predicted by software DNAMAN V6.0 using PAT-1 domain sequences. ADRP: cattle (NM_173980), dog (XP_531946), pig (NP_999365), human (NP_001113), mouse (NP_031434), chicken (XP_424822), zebrafish (NP_001025433). TIP47: cattle (ABG67022), pig (NP_001026948), human (NP_005808), dog (XP_542149), mouse (NP_080112), frog (AAH46724). PLIN: cattle (XP_598845), dog (XP_545853), human (NP_002657), mouse (AAN77870), pig (NP_001033727), chicken (XP_413860).

database http://www.threakdb.org). Since both *SW0170* [6] and *ADM* [12] have previously been FISH-mapped to SSC2p11, we could deduce that *M6PRBP1* is localised in this region *i.e.* SSC2p11.

Human *PLIN* and *M6PRBP1* genes are located on chromosomes 15q26 [23] and 19p13.3 (NM_005817), respectively. Thus, our result is consistent with the pig/human comparative map and supports the conservation of synteny between SSC7 and HSA15 and between SSC2 and HSA19 [8].

3.3. Tissue expression patterns of porcine *PLIN*, *ADRP* and *M6PRBP1* genes

The relative transcription levels of porcine *PLIN*, *ADRP* and *M6PRBP1* are shown in Figures 2a and 2b. Porcine *PLIN* mRNA is ubiquitously expressed,

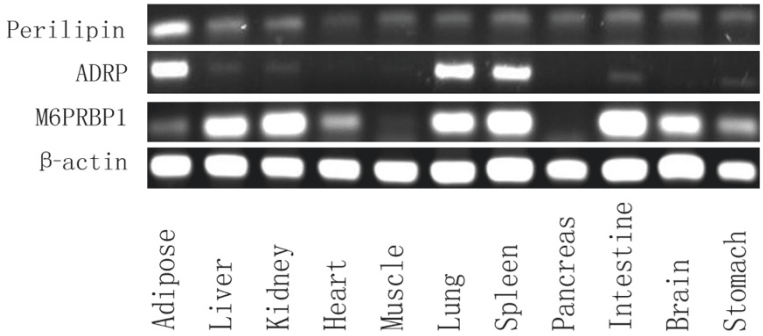

Figure 2a. Expression of *PLIN*, *ADRP* and *M6PRBP1* mRNAs in porcine tissues. RT-PCR results for porcine *PLIN*, *ADRP*, *M6PRBP1* and β-actin mRNAs. PCR of *PLIN*, *ADRP* and *M6PRBP1* mRNAs comprised 31 cycles and that of β-actin 28 cycles.

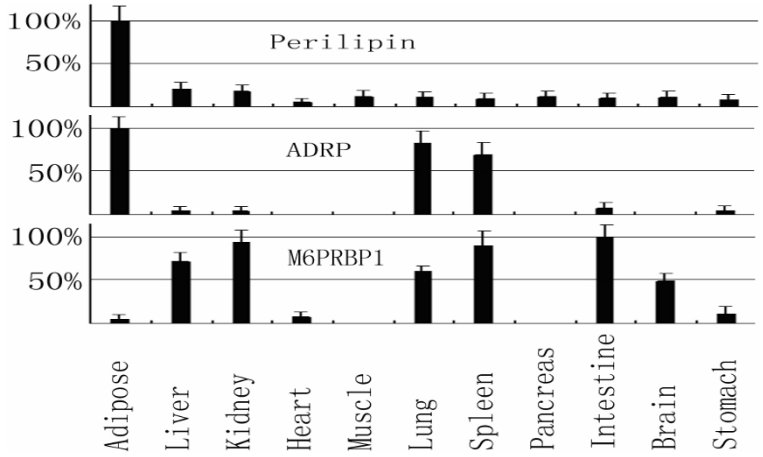

Figure 2b. Comparison of mRNA levels of *PLIN*, *ADRP* and *M6PRBP1* in various tissues. The level of β-actin mRNA was used to normalize those of *PLIN*, *ADRP* and *M6PRBP1*. The bar shows the relative values in different tissues taking that of adipose tissue as 100% for *PLIN* and *ADRP* and that of intestine as 100% for *M6PRBP1*. The experiment was performed in three pigs and each value was expressed as the mean value ± Standard Deviation (SD).

with the highest level found in adipose tissue and a low level in all other tissues. *ADRP* mRNA is highly expressed in adipose tissue, lung and spleen and less in liver, kidney, intestine and stomach. *M6PRBP1* mRNA is highly expressed in intestine and at decreasing levels in kidney, spleen, liver, lung, brain, adipose tissue, heart and stomach.

It has been suggested that the expression of *PLIN* is restricted to adipocytes and steroidogenic cells [5]. These cell classes have a mechanism

for the lipolysis of stored triacylglycerol (TAG) that is mediated by cAMP-dependent protein kinase A (PKA) and hormone-sensitive lipase (HSL). In our analysis, porcine *PLIN* mRNA was found to be highly expressed in adipose tissue, but also ubiquitously expressed in the other tissues at a very low level. This may be explained by the fact that these tissues contain few adipocytes and steroidogenic cells (for example liver and kidney). In fact, we have also found that the porcine HSL is ubiquitously expressed in different tissues (data not shown), which indicates that the lipolysis of stored TAG, which is mediated by PKA and HSL, is not restricted to adipose tissue.

Porcine *M6PRBP1* mRNA is highly expressed in intestine, kidney, spleen, liver and lung, but much less in adipose tissue. Although the content of lipid droplets in intestine is quite high, it does not exceed that in the adipose tissue. This may contribute to the fact that M6PRBP1 associates not only with lipid droplets, but also with the mannose 6-phosphate receptor [2].

Although the levels of *PLIN*, *ADRP* and *M6PRBP1* mRNA are different in various tissues, total levels are higher in tissues with a high lipid content (adipose tissue, liver, kidney, lung, spleen, intestine) than in tissues with a low lipid content (heart, muscle, pancreas and stomach). The distribution of PAT mRNAs is consistent with lipid levels in different tissues, thus they can be considered as a marker of lipid accumulation.

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