

# Differential expression of *ATPAF1* and *ATPAF2* genes encoding $F_1$ -ATPase assembly proteins in mouse tissues

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**Abstract** Atp11p (Atpaf1p;  $F_1$ -ATPase assembly factor 1) and Atp12p (Atpaf2p;  $F_1$ -ATPase assembly factor 2) are proteins required for the assembly of  $\beta$  ( $F_1$ - $\beta$ ) and  $\alpha$  ( $F_1$ - $\alpha$ ) subunits into the mitochondrial ATPase. Here we report about 100 times lower levels of *ATPAF1* and *ATPAF2* transcripts in relation to the mRNA levels of  $F_1$ - $\alpha$  and  $F_1$ - $\beta$  in a range of mouse tissues. Quantitative reverse-transcription polymerase chain reaction revealed nearly constant *ATPAF1* expression in all tissues in both adult and 5-day-old mice (up to two-fold differences), indicating that *ATPAF1* rather behaves like a maintenance gene. In contrast, *ATPAF2* expression differed up to 30-fold in the tissues analysed. *ATPAF2* tissue-specific expression was also found to correlate well with mRNA levels of both  $F_1$ - $\alpha$  and  $F_1$ - $\beta$  (BAT  $\gg$  kidney, liver  $>$  heart, brain  $>$  skeletal muscle), showing the highest mRNA level in the thermogenic, ATPase-poor brown adipose tissue, which is characterised by a 10-fold decrease in ATPase/respiratory chain stoichiometry relative to the other tissues.

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**Key words:** Mitochondrial ATPase; Assembly; *ATPAF1*; *ATPAF2*; Gene expression; Brown adipose tissue

## 1. Introduction

Mammalian ATP synthase (ATPase) is an oligomeric enzyme consisting of a catalytic  $F_1$  moiety which is connected to the proton-translocating component ( $F_0$ ) via central and periphery stalks [1]. The  $F_1$  part is comprised of five nuclear-coded subunits that are present in the stoichiometric ratio,  $\alpha_3\beta_3\gamma\delta\epsilon$  [2]. The  $F_0$  sector is composed of 11 different subunits, which are encoded either by nuclear or mitochondrial DNA [1]. Therefore, the biosynthesis of ATPase is a complex process dependent on a coordinate expression from both genomes, import machinery and the assembly pathway itself.

Studies in yeast identified five chaperone-type factors necessary for assembly of the functional enzyme. Atp10p and Atp22p were found to mediate  $F_0$  assembly [3,4], while

Atp11p, Atp12p and Fmc1p are required for the  $F_1$  part [5,6]. Fmc1p is essential at elevated growth temperatures but *FMCI* deletion can be rescued by overexpression of Atp12p [6]. Recently, human orthologues of Atp11p and Atp12p (Atpaf1p and Atpaf2p according to gene nomenclature) were also identified. Like the yeast proteins, human Atp11p has chaperone-like activity towards the  $\beta$  subunit and human Atp12p has been proposed to interact with the  $\alpha$  subunit [7]. Genes coding for Atp11p and Atp12p seem to be generally present in eukaryotes [7,8]; however, it is not clear whether besides the chaperone activity they could also be involved in the regulation of the mitochondrial content of ATPase.

An interesting mammalian model for studying ATPase assembly factors could be provided by brown adipose tissue, which has a high content of mitochondria but about 10 times lower amount of ATPase in comparison with respiratory chain enzymes [9–11]. Biosynthesis of the c subunit of the  $F_0$  channel and the expression of the *cPI* gene in particular determines the amount of ATPase in brown adipose tissue and most probably in other tissues [12–14]. Additional control mechanisms have been proposed, involving expression of tissue-specific isoforms of the  $\gamma$  subunit [15,16] and transcriptional and post-transcriptional regulation of  $\beta$  subunit expression [17–20].

The aim of the present study was to evaluate how *ATPAF1* and *ATPAF2* genes are expressed in mammalian tissues and how their expression is related to the mRNA levels for corresponding  $F_1$  subunits,  $\alpha$  and  $\beta$ , with which they are supposed to interact.

## 2. Materials and methods

### 2.1. Animals

Mice of Balb/c inbred strain were kept at 22°C and 12-h light/12-h dark cycle with standard diet and water ad libitum. Both 2-month-old adult and 5-day-old young mice were killed by cervical dislocation and tissue samples were frozen in liquid nitrogen and stored at –70°C until RNA extraction.

### 2.2. Northern blot analysis

RNA Blue reagent (Top-Bio, Czech Republic) was used for total RNA isolation, polyA<sup>+</sup> RNA was isolated from human muscle obtained from orthopaedic operation using the Micro-Fast Track 2.0 kit (Invitrogen). RNA was separated on a 1.25% (w/v) agarose gel (containing 20 mM MOPS pH 7.0, 6.7% (v/v) formaldehyde, 50 mM sodium acetate and 10 mM EDTA) and was transferred to a Hybond-N membrane (Amersham Biosciences, UK) by capillary blotting overnight. The membrane was prehybridised at 42°C for 2 h (5× SSC pH 7.0, 5× Denhardt's, 0.5% (w/v) SDS, 50 mM sodium phosphate pH 6.5, 50% (v/v) formamide and 100 µg/ml herring sperm DNA), hybrid-

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**Abbreviations:** ATPase, ATP synthase;  $F_1$ , catalytic part of ATPase; Atp11p (Atpaf1p),  $F_1$ -ATPase assembly factor 1; Atp12p (Atpaf2p),  $F_1$ -ATPase assembly factor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Table 1  
Primers for real-time RT-PCR analysis

mRNA	Accession number	Primer sequences (5'–3')		Product bp
		Forward	Reverse	
<i>ATPAF1</i>	XM_204176	CAGTGCATCGCCAATCAGGTTTCAG	CGTTTCAGTTCGGCTCCAAGTCCA	155
<i>ATPAF2</i>	NM_145427	AGCATCCCTACCCAGACCCGAGAG	CAATCAGGCCCAAAGTCAGCAACA	127
<i>F<sub>1</sub>-α</i>	NM_007505	TATGCGGGTGTACGGGGTTATCTT	TGAGCTTTGCGTCTGACTGTTCTG	157
<i>F<sub>1</sub>-β</i>	NM_016774	GCAGGAAAGGATCACCACCACCAA	CAGCAATAGCCCGGGACAACACAG	146
<i>GAPDH</i>	M32599.1	GAGCGAGACCCCACTAACATCAAA	TGGGGGCATCGGCAGAAGG	154

isation was carried out overnight at 42°C in the same solution containing [<sup>32</sup>P]dATP-labelled cDNA, labelled by random priming (Ambion, USA). Blots were hybridised to cDNA probes corresponding to the 341 bp fragment of human *F<sub>1</sub>-β* (NM\_001686, bases 870–1210), the 474 bp fragment of human *ATPAF1* (BC\_008498, bases 1070–1544) and the 639 bp fragment of human *ATPAF2* mRNA (NM\_145691.2, bases 217–855). The radioactive signal was quantified using the BAS-5000 system (Fuji, Japan). Blots were stripped and rehybridised sequentially.

### 2.3. Quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Primers (Table 1) were designed using the PrimerSelect software (version 4.04, DNASTAR Inc.). 2 µg of total RNA was reverse-transcribed in 20 µl reaction with SuperScript II (Invitrogen). Quantitative RT-PCR was performed on a LightCycler (Roche) in a 10 µl volume with 0.5 µM primer concentration. HotStarTaq DNA polymerase, buffer, nucleotides and MgCl<sub>2</sub> were included in the QuantiTect SYBR Green PCR kit (Qiagen). 2.5 mM MgCl<sub>2</sub> was found to be optimal for all primer pairs. Initial 15 min denaturation at 95°C to activate the HotStarTaq polymerase was followed by 45 cycles with a 94°C denaturation for 15 s, 60°C annealing for 25 s and 72°C extension for 20 s. Fluorescent product was detected at the end of each elongation phase. Specificity of the PCR was confirmed by melting curve analysis (95°C for 0 s, cooling down to 68°C for 15 s, heating to 95°C at a rate of 0.1°C/s) and agarose gel electrophoresis (Fig. 1). Standard curves were generated after serial dilutions of a cDNA obtained from adult heart. Quantification data were obtained in triplicate from two or three independent RNA isolations and analysed by Second Derivative Maximum Method with the LightCycler analysis software (version 3.3, Roche). Pooled tissues of three animals were used for each RNA isolation from heart and brown adipose tissue from young mice. Levels of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) mRNA were used to correct inter-sample variation.

## 3. Results

### 3.1. ATPAF1 and ATPAF2 transcripts are low-abundant

Northern blot analysis of total RNA from human muscle, rich in the content of ATPase, revealed a specific but very weak signal of both *ATPAF1* and *ATPAF2* mRNAs. This signal could be increased by using polyA<sup>+</sup> RNA but its com-

parison with the signal of *F<sub>1</sub>-β* mRNA clearly indicated that *ATPAF1* and *ATPAF2* genes are expressed at a much lower level (Fig. 2). Thus, in further experiments, we used quantitative RT-PCR analysis to examine *ATPAF1* and *ATPAF2* mRNA levels in different mouse tissues simultaneously with *F<sub>1</sub>-α* and *F<sub>1</sub>-β* transcripts.

### 3.2. Expression of genes encoding α and β subunits of the catalytic moiety of ATPase

The expression of *F<sub>1</sub>-α* and *F<sub>1</sub>-β* was analysed in several mouse tissues with high energy demands (Fig. 3), namely in heart, kidney, brown adipose tissue and liver from both adult and young animals. In addition, muscle and brain from adult mice were also used. *F<sub>1</sub>-α* and *F<sub>1</sub>-β* transcripts were normalised to the level of *GAPDH* mRNA [21] and results were expressed relatively to the value from adult heart, defined as 1. RT-PCR revealed tissue-specific differences in *F<sub>1</sub>-α* mRNA levels within the range of 0.3–2.0, and similarly the content of *F<sub>1</sub>-β* transcript varied between 0.3 and 2.8 in adult tissues. *F<sub>1</sub>-α* and *F<sub>1</sub>-β* tissue profiles were alike, with the highest mRNA levels observed in brown adipose tissue and the lowest in muscle, resulting in seven- to nine-fold difference in both mRNA levels. The same expression profiles of *F<sub>1</sub>-α* and *F<sub>1</sub>-β* were found in 5-day-old mice. Resulting *F<sub>1</sub>-α*/*F<sub>1</sub>-β* mRNA ratios ranged between 0.6 and 1.0 in all tissues tested and clearly confirmed the similarity between *F<sub>1</sub>-α* and *F<sub>1</sub>-β* expression patterns.

### 3.3. ATPAF1 expression even contrasts with variable expression of ATPAF2, which is the highest in brown adipose tissue

We further quantified the mRNA levels of *ATPAF1* and *ATPAF2*, which were also normalised to *GAPDH* transcript (Fig. 4). *ATPAF1* mRNA levels remained steady in all tissues and did not exceed a two-fold difference in adults or the young. Conversely, *ATPAF2* mRNA levels were much more

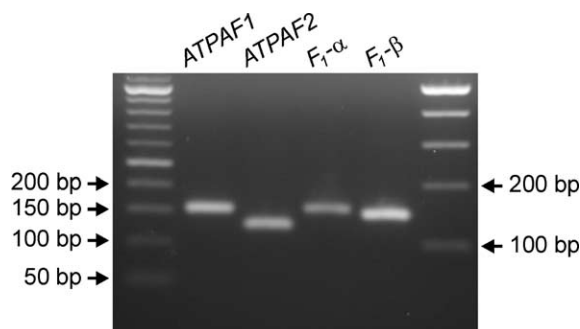


Fig. 1. Agarose gel electrophoresis of *ATPAF1*, *ATPAF2*, *F<sub>1</sub>-α* and *F<sub>1</sub>-β* PCR products.

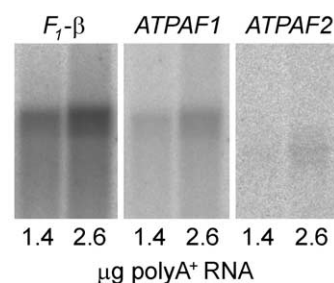


Fig. 2. Northern blot analysis of *ATPAF1*, *ATPAF2* and *F<sub>1</sub>-β* mRNAs. Indicated amounts of polyA<sup>+</sup> RNA from human muscle were used.

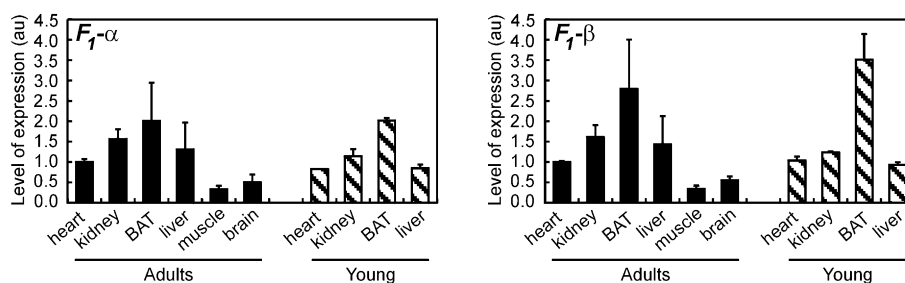


Fig. 3. Tissue expression profile of  $F_1\text{-}\alpha$  and  $F_1\text{-}\beta$  mRNAs in adult and 5-day-old mice analysed by quantitative RT-PCR. Transcript levels are normalised to *GAPDH* mRNA and are expressed relative to the mean value from adult heart, which was set to 1. Data are means  $\pm$  S.D.; au, arbitrary units; BAT, brown adipose tissue.

variable, and likewise  $F_1\text{-}\alpha$  and  $F_1\text{-}\beta$  transcripts, reached a maximum in brown fat, were considerably lower in kidney and liver, decreased further in heart and brain and were the lowest in muscle. *ATPAF2* mRNA levels ranged between 0.6 and 17 in the tissues tested, which corresponded to a 30-fold difference. Interestingly, the expression in brown fat was about one order of magnitude higher than in other tissues; in three independent experiments we found a 14–21-fold higher level of *ATPAF2* mRNA in adult brown fat than in heart, and a 9–10-fold difference was present in young animals. Consequently, the resulting *ATPAF2/ATPAF1* ratios in mouse tissues mirrored the expression profile of *ATPAF2*.

Quantitative RT-PCR further confirmed a large excess of  $F_1\text{-}\alpha$  and  $F_1\text{-}\beta$  mRNAs in relation to *ATPAF1* and *ATPAF2* transcripts. Analysis of crossing points suggested 1.9 and 2.3 orders of magnitude lower levels of *ATPAF1* and *ATPAF2* transcripts in comparison with  $F_1\text{-}\beta$  and  $F_1\text{-}\alpha$  mRNAs, respectively. Even in the case of brown fat, there is about a 10-fold difference between *ATPAF2* and  $F_1\text{-}\alpha$  mRNA levels.

#### 4. Discussion

Our quantitative analysis of *ATPAF1* and *ATPAF2* transcripts revealed about 100 times lower expression in comparison to  $F_1\text{-}\alpha$ ,  $F_1\text{-}\beta$  and *GAPDH* genes in various mouse tissues. Several observations support either qualitatively or quantitatively these results: Northern blot detection of assembly proteins mRNAs gave a poor signal in relation to  $F_1\text{-}\beta$  mRNA (this study); similarly Northern blot analysis of poly-A<sup>+</sup> RNA with a radioactive probe to 3'-UTR of *ATPAF2* resulted in a barely visible signal in human tissues in comparison with the expression of several other genes located on chromosome 17p11.2 [22]. Furthermore, Serial Analysis of Gene Expression (SAGE [23]) data, which are available via

a public resource (<http://www.ncbi.nih.gov/SAGE/> [24]), showed the following levels of expression in normal human tissues: *ATPAF1*, *ATPAF2* <  $F_1\text{-}\alpha$ ,  $F_1\text{-}\beta$  < *GAPDH* with approximately 10-fold difference in expression level between adjacent groups. Our quantitative RT-PCR studies on human fibroblasts (data not shown) were in good agreement with SAGE results, which unfortunately cannot be obtained for *ATPAF1* and *ATPAF2* transcriptional abundance in mouse tissues due to the lack of data in the resource. Nonetheless, SAGE of  $F_1\text{-}\alpha$ ,  $F_1\text{-}\beta$  and *GAPDH* expression levels in mouse tissues have led to the same conclusion as was reached in the present study.

Northern blot analysis has been mainly used to study the expression of  $F_1\text{-}\alpha$  and  $F_1\text{-}\beta$  in mammalian tissues so far. High similarity between  $F_1\text{-}\alpha$  and  $F_1\text{-}\beta$  expression profiles and constant  $F_1\text{-}\alpha/F_1\text{-}\beta$  mRNA ratios in mouse tissues including slightly higher  $F_1\text{-}\beta$  expression in brown adipose tissue relatively to  $F_1\text{-}\alpha$  mRNA are consistent with previous studies [12]. Only small differences in levels of  $F_1\text{-}\beta$  between adults and the young were observed in brown adipose tissue of mice [19] or in rat liver [25]. Some authors reported significantly higher expression of  $F_1\text{-}\alpha$  or  $F_1\text{-}\beta$  in heart compared to several other tissues where content of  $F_1\text{-}\alpha$  or  $F_1\text{-}\beta$  transcripts varied [14,26,27]. Some discrepancies between individual studies may be in the way of data evaluation. There is a general tendency to correct inter-sample variation by the use of an internal standard whose mRNA levels should not vary under the experimental conditions used [28]. *GAPDH* was considered as a suitable reference for expression analysis in human tissues [21]. Accordingly, we did not see significant variation of *GAPDH* expression in samples from the same starting amount of RNA. Moreover, the use of  $\beta$ -actin as a reference gene resulted in similar expression profiles (data not shown). Despite the differences among individual studies, an apparent

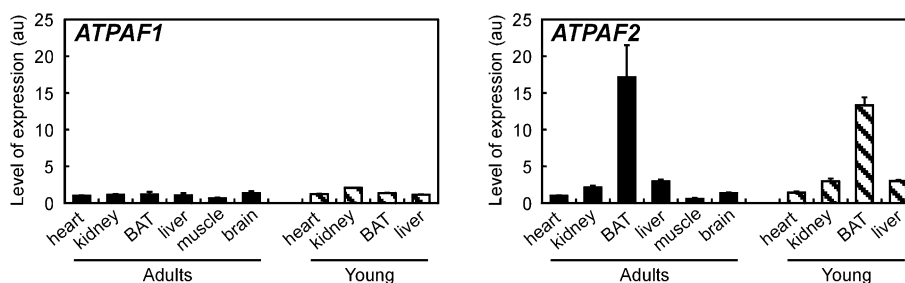


Fig. 4. Tissue expression profile of *ATPAF1* and *ATPAF2* mRNAs in adult and 5-day-old mice analysed by quantitative RT-PCR. Transcript levels are normalised to *GAPDH* mRNA and are expressed relative to the mean value from adult heart, which was set to 1. Data are means  $\pm$  S.D.; au, arbitrary units; BAT, brown adipose tissue.

Table 2  
Selection of predicted transcription regulatory sites in *ATPAF2* promoters

Element	Gene	Genomic contig	Locus link number	Position from ATG	Strand	Sequence
E-box	<i>hATPAF2</i>	NT_010718	91 647	–312 to –298	–	GCGCCACGTGTTTTC
				–255 to –241	–	CCTCCACGCCGCGCC
	<i>mATPAF2</i>	NT_039520	246 782	–349 to –335	+	AGCTCAGCGGCGCT
				–314 to –300	–	GCGCCACGTGTTTTC
	<i>rATPAF2</i>	NW_042658	303 190 <sup>a</sup>	–348 to –334	+	AGCTCAGCGGCGCT
				–313 to –299	–	GCACCACGTGTTTTC
Sp1/GC	<i>hATPAF2</i>	NT_010718	91 647	–243 to –229	+	AGGGGGCGGGGCGAG
	<i>mATPAF2</i>	NT_039520	246 782	–245 to –231	+	AGGGGGCGTGGCGAG
	<i>rATPAF2</i>	NW_042658	303 190 <sup>a</sup>	–244 to –230	+	AGGGGGCGGGGCGAG
YY1	<i>hATPAF2</i>	NT_010718	91 647	–156 to –138	–	CGCCGCCATCTTCGCATG
	<i>mATPAF2</i>	NT_039520	246 782	–152 to –134	–	CGCCGCCATCTTCCTCATG
	<i>rATPAF2</i>	NW_042658	303 190 <sup>a</sup>	–152 to –134	–	CGCCGCCATCTTCGTCATG

*h*, human; *m*, mouse; *r*, rat.

<sup>a</sup>Original 5' was incorrect and had to be found within the genomic contig based on sequence similarity to mouse and human genes.

analogy between *F<sub>1</sub>-α* and *F<sub>1</sub>-β* expression that we found was not at all affected by correlation to a reference gene.

The tissue distribution of *ATPAF2* transcript closely resembles those of *F<sub>1</sub>-α* and *F<sub>1</sub>-β* and is in sharp contrast to the nearly constant *ATPAF1* mRNA level. This suggests that *ATPAF1* behaves more like a maintenance gene, which serves the cell with a constitutive expression of the chaperone. *ATPAF2*, *F<sub>1</sub>-α* and *F<sub>1</sub>-β* transcription might be, at least partly, under a similar control mechanism, which is probably not the control step of ATPase content as indicated by the high transcripts' content in brown fat. Nonetheless, mRNA level is not necessarily the indicator of protein level because mRNA stability, half-time, subcellular localisation and resulting translational efficiency are subjected to a complex regulation in mammalian cells. Indeed, high mRNA levels of most ATPase subunits in brown fat contrast with low ATPase content compared to other tissues [12,17].

Promoters of *F<sub>1</sub>-α* and *F<sub>1</sub>-β* genes differ and are known to be controlled by distinct regulatory factors, which are somehow effective in uniformly regulating the transcriptional activity of these genes. USF2 [29], YY1 [30] and COUP-TFII/ARP-1 [31] represent experimentally verified *F<sub>1</sub>-α* *trans*-acting factors. Transcription control of the *F<sub>1</sub>-β* gene involves a proximal CCAAT box essential for basal expression [32,33], a far-upstream enhancer [34], an OXBOX/REBOX element involved in myogenic cell expression [33] and a close upstream Ets domain with an adjacent GC-rich region facilitating the binding of NRF2/GABP and Sp1, respectively [19].

As no theoretical or functional studies of transcriptional control of *ATPAF1* and *ATPAF2* genes are available yet, we used GenomatixSuite software (<http://www.genomatix.de>) for prediction of transcription factor binding sites in putative promoters of human, mouse and rat *ATPAF1* and *ATPAF2* genes. Both genes are devoid of TATA-box and TATA-less promoters were identified for example for *F<sub>1</sub>-α* [35], *F<sub>1</sub>-β* [26], *F<sub>1</sub>-γ* [36] and cytochrome *c* oxidase subunit IV genes [37]. Proposed modules in *ATPAF1* promoters did not contain any transcription binding site known to be involved in the regulation of mammalian mitochondrial biogenesis [38]. On the other hand, *ATPAF2* genes seem to share potential regulatory elements with genes coding for ATPase subunits and other mitochondrial proteins (Table 2). Predicted E-box motifs could provide binding sites for USF [39]. Sp1 and YY1 are other elements that might play a regulatory role in *ATPAF2* expression. Sp1 has been shown to be involved in the

regulation of *F<sub>1</sub>-β*, *ANT2*, and cytochrome *c* oxidase genes; similarly, YY1 can have a profound effect on cytochrome *c* oxidase biogenesis (for review see [38]).

In conclusion, *ATPAF1* and *ATPAF2* genes differ significantly in their expression profiles. *ATPAF2* expression resembles the expression patterns of *F<sub>1</sub>-α* and *F<sub>1</sub>-β* genes with the following tissue distribution: BAT ≫ kidney, liver > heart, brain > skeletal muscle. *ATPAF1* shows constant expression in the tissues tested, which is a feature of housekeeping genes. Unlike *ATPAF1*, *ATPAF2* possibly shares transcriptional regulatory elements common to ATPase or other mitochondria-related genes.

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