Genomic organization of the mouse peroxisome proliferator-activated receptor β/δ gene: alternative promoter usage and splicing yield transcripts exhibiting differential translational efficiency

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Peroxisome proliferator-activated receptor (PPAR) β/δ is ubiquitously expressed, but the level of expression differs markedly between different cell types. In order to determine the molecular mechanisms governing PPARβ/δ gene expression, we have isolated and characterized the mouse gene encoding PPARβ/δ. The gene spans approx. 41 kb and comprises 11 exons of which the six exons located in the 3′-end of the gene are included in all transcripts. Primer-extension and 5′-rapid amplification of cDNA ends experiments revealed the presence of multiple transcription start points and splice variants, originating from the use of at least four different promoters. One of these transcription start points was found to be used predominantly in all tissues examined. Initiation from this major transcription start point gives rise to a transcript with a 548 nt 5′-untranslated leader containing eight upstream AUG codons. We show that the presence of the 548 nt leader resulted in a low translational efficiency of the corresponding PPARβ/δ mRNA and propose, based on structural features of the 5′-untranslated region, that translational initiation may be mediated via an internal ribosome entry site-dependent mechanism.

Key words: expression, nuclear hormone receptor, translation.

INTRODUCTION

The peroxisome proliferator-activated receptor (PPAR) subfamily of the nuclear hormone receptors comprises three distinct subtypes found in vertebrate species, PPARα, PPARβ/δ [also named nuclear hormone receptor 1 (NUC) or fatty acid-activated receptor (FAAR)] and PPARγ. They function as ligand-activated receptors and regulators of transcription responding to a wide range of ligands, including fatty acids, eicosanoids, fibrates and thiazolidinediones.

Whereas the importance of PPARα and PPARγ in the control of lipid homeostasis and cellular growth and differentiation have been well established, the role of PPARβ/δ remained elusive until recently, where a number of studies have implicated PPARβ/δ in various processes. Thus PPARβ/δ has been proposed to be involved in the differentiation of oligodendrocytes [1], epithelial cells [2,3], keratinocytes [4] and adipocytes [5–7]. PPARβ/δ seems also to play an important role in lipid metabolism in the brain [8], in the regulation of uncoupling protein 3 levels in myotubes [9], in cholesterol homeostasis [10,11] and in bone metabolism [12]. Moreover, recent data suggest important roles for PPARβ/δ in skin wound healing [13], and in embryo implantation and decidualization [14]. PPARβ/δ-null mice also have altered myelination in the central nervous system, reduced adipose stores and changes in the response to PMA-induced inflammation and hyperproliferation of the epidermis [8,15]. Finally, a role in the initiation of colon cancer has been suggested [16–18], although recent data indicate that PPARβ/δ is dispensable for polyp formation in APC(min) mice [19].

The genomic structures of the genes encoding mouse PPARα and PPARγ have been described [20,21], and here we present a detailed analysis of the genomic structure of the gene encoding mouse PPARβ/δ. The mouse PPARβ/δ gene contains a total of 11 exons; the six exons in the 3′-end encode the PPARβ/δ protein. The organization of the coding exons of PPARβ/δ corresponds to that of the genes encoding PPARα and PPARγ. Compared with the human PPARβ/δ gene [22] we noticed several differences in the 5′-region of the mouse gene, where the number and organization of exons appear to differ in the two species. Alternative promoter usage gives rise to four differentially spliced versions of PPARβ/δ mRNAs containing one or two non-coding 5′-exons, and we present evidence suggesting that PPARβ/δ expression is regulated at the post-transcriptional level. The transcript found to be most abundantly expressed in the tissues examined has features in common with mRNAs depending on internal ribosome entry site (IRES)-mediated translational initiation and post-transcriptional regulation, and we propose that the initiation of translation of this prominent PPARδ transcript may be accomplished via an IRES-dependent mechanism.

EXPERIMENTAL

Genomic cloning and Southern analysis

A mouse genomic library in λ FIX™ II was screened using ^{32}P-labelled PPARβ/δ mRNA [23,24]. Positive clones were isolated and purified by three successive rounds of screening. Three genomic P1 clones were obtained from Genome Systems (St. Louis, MO, U.S.A.) after PCR screening with the primers 5′-CCTGCAGGCCTGGTCATAGC-3′ and 5′-GGACAGCTGCGTCCTGAG-3′. The clones were further screened by PCR with the primer sets 5′-GGATTCTGCGGAGCGTGCG-3′/5′-CTGTCAGTGAGCAGGAGCCTC-3′ and 5′-GGCGACCTGGGGATTAATG-3′/5′-CTGTCCCGGCCGCCTCTC-3′. One of these clones, plate 91, was used for the preparation of

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; IRES, internal ribosome entry site; M-MLV, Moloney-murine-leukaemia virus; PPAR, peroxisome proliferator-activated receptor; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

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The nucleotide sequence for the gene encoding PPARβ/δ has been submitted to the EMBL Nucleotide Sequence Database under accession numbers AJ420918, AJ420919, AJ420920, AJ420921 and AJ420922.

genomic subclones harbouring exons 1B, 1C and 2 by partial cutting with *Sau*3AI and cloning of the fragments into the *Bam*HI site of pBluescript KS+ (Stratagene). Subclones harbouring these exons were found by colony hybridization using the oligonucleotide probes 5′-CTGTCCCGGCCGCCTCTC-3′ and 5′-CTGTCAGTGAGCAGGAGCCTC-3′, respectively. The P1 clone was used as a template for obtaining genomic clones containing exon 1A and 1D by long-range PCR using the BioExact enzyme mixture (Bioline). Here the primer pairs 5′-GGCCGTCGACATTTAGGTGACAC-3′ / 5′-AGGGCTCAGGAGCGGCTTC-3′ and 5′-GGGGCTGCTTGCTAAATAGG-3′/5′-CAGTCCTGTCTCCAGATCTG-3′ were employed for the PCR amplifying exons 1A and 1D, respectively. The resultant fragments were cloned into pCRII TOPO. Genomic subclones covering exons 3–8 were generated by cutting the λ clones with *Xba*I and cloning of the resulting fragments into the *Xba*I site of pBluescript KS+.

Sequencing was performed on an A.L.F. (Pharmacia) or a CEQ2000 (Beckman-Coulter) sequencer except a short GC-rich stretch in exon 1B that proved impossible to sequence by conventional methods and therefore was sequenced by MS as described elsewhere [25]. All intron–exon boundaries were sequenced. Most intron sequences were also determined. The obtained sequences were analysed and assembled by programs from the GCG package (Genetics Computer Group, Madison, WI, U.S.A.). Genomic Southern blotting was performed by standard procedures using as a probe a 386 bp fragment generated by PCR on a genomic clone containing the 3′-end of the gene with the primers 5′-CAGAGAGCAAGTGGGCAG-3′ and 5′-AGGTGTGCCAGCATCTCCC-3′.

Primer extension

RNA (10 μg) in annealing buffer (250 mM KCl/10 mM Tris/HCl, pH 8.3, at 42 °C) was annealed with 0.5 pmol of ^{32}P-labelled primer [exon 1A, 5′-AGGGCTCAGGAGCGGCTTC-3′ (50 °C); exon 1B, 5′-TTCCCATTAATCCCCAGGTC-3′ (45 °C); exon 1C, 5′-GAATCCAACATTGAAGGCCCA-3′ (50 °C); exon 1D, 5′-CAGTCCTGTCTCCAGATCTG-3′ (50 °C)] for 1 h at the temperature indicated, followed by the addition of 14 μl of extension mixture [66 mM Tris/HCl, pH 8.3, at 42 °C, 4 mM $MgCl_2$, 13.3 mM dithiothreitol, 0.66 mM dNTP, 14.3 units/μl Moloney-murine-leukaemia virus (M-MLV) reverse transcriptase (Gibco BRL) and 133 μg/ml actinomycin D] and extension for 1 h. The reaction was terminated by addition of EDTA to 10 mM, and the samples were dried, redissolved in formamide dye mix and run on a denaturing gel. Sets of corresponding sequencing reactions using the same primers were run alongside the samples as markers.

5′-Rapid amplification of cDNA ends (RACE)

Two first-strand synthesis reactions were set up as outlined in the Capfinder kit (Clontech) protocol using 1 μg of liver RNA isolated from a C57 Bl/6J mouse and the CDS/3′ primer provided in the kit, or a gene-specific primer (5′-TCTCCTCCTGTGGCTGTTCCATGACTGACC-3′). The first-strand product was used as a template in a PCR with the 5′-PCR primer provided in the kit and the gene-specific primer using *Pfu* DNA polymerase. The product was purified on a High-pure column (Roche) and reamplified using the 5′-PCR primer provided in the kit and a nested gene-specific primer, (5′-CCTGCAGGCCTGGTCATAGC-3′). The resulting products were cloned into pBluescript KS+ and analysed by sequencing.

Cell culture

3T3-L1 preadipocytes were grown to confluence and induced to differentiate as described in [26]. Briefly, preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum and, 2 days after reaching confluence (day 0), differentiation was induced by culturing the cells in DMEM with 10% fetal calf serum, 167 nM insulin, 0.5 mM methylisobutylxanthine and 0.25 μM dexamethasone for 2 days, DMEM with fetal calf serum and insulin for 2 days and DMEM with fetal calf serum until RNA was isolated.

3T3-F442A preadipocytes were grown to confluence and induced to differentiate to adipocytes as described previously [23]. Briefly, preadipocytes were cultured in DMEM supplemented with 10% fetal bovine serum (termed standard medium) and after reaching confluence (day 0), differentiation was induced by culturing cells in the same medium containing 17 nM insulin and 2 nM tri-iodothyronine (termed differentiation medium). Media were changed every other day. HEK-293 cells were maintained in standard medium.

Transient transfections

The following promoter fragments were cloned into pGL3 Basic (positions relative to the A in the AUG start codon in the spliced mRNA): 1A, approx. −4000 to −72 in the 1A promoter; 1B3/4, −393 to −172 in the 1B promoter; 1B1-4, −1089 to −256 in the 1B promoter; 1C, −2786 to −188 in the 1C promoter; 1D, approx. −2400 to −85 in the 1D promoter. The promoter activity was evaluated by performing transfections with the FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Typically, for four wells of a 24-multiwell culture plate, 2 μg of luciferase construct was co-transfected with 50 ng of pCMV-β-galactosidase, a control vector used to monitor transfection efficiency. Luciferase construct used in this range of concentrations leads to saturating conditions where the result is largely independent of the DNA concentrations in the range used here (results not shown). DNA in 100 μl of DMEM was complexed with 3 μl of FuGENE 6 for 15 min before addition of 2 ml of either standard or differentiation medium. The cells were incubated in the presence of the transfection mixture (0.5 ml/well) for 6–8 h, and were then maintained in standard or differentiation media for a further 36 h before lysis for reporter assays.

Luciferase and β-galactosidase activities were determined in the same cell extract using a Berthold luminometer and commercial kits. Luciferase activities were determined in quadruplicate and normalized to β-galactosidase activities. The experiment was performed three times.

RNA isolation and Northern analysis

Total RNA was isolated from the cultured cells at various time points during the differentiation process or from tissues isolated from male C57 black mice and subjected to Northern analysis. Filters were stained with Methylene Blue to confirm equal loading and hybridized to DNA probes labelled with ^{32}P by random primer extension.

Western blotting

Western blotting was performed as described in [27]. PPARβ/δ antiserum was obtained from Walter Wahli (Institut de Biologie Animale, Universite de Lausanne, Lausanne, Switzerland). The bands containing PPARβ/δ were identified by co-migration with PPARβ/δ obtained from NIH-3T3 cells transfected with a construct encoding full-length PPARβ/δ.

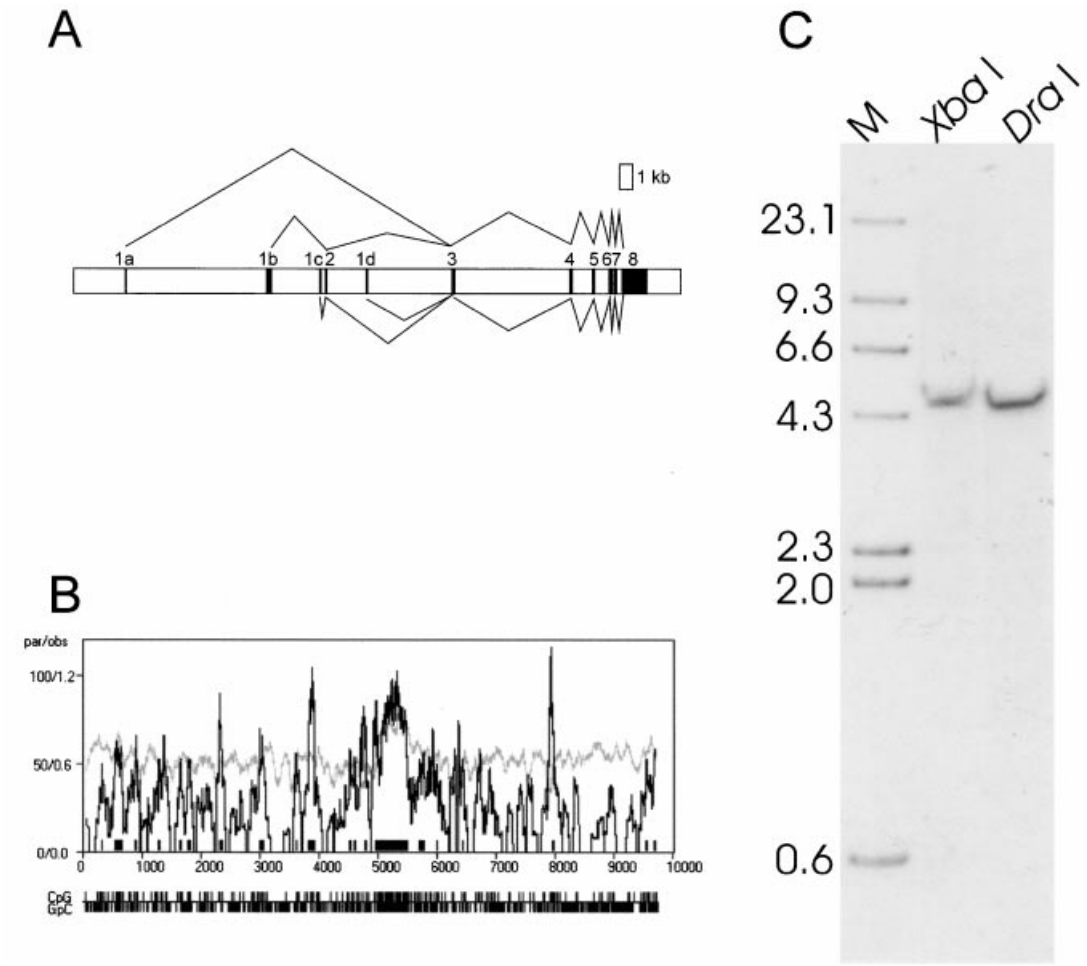

Figure 1 Structure of the mouse PPARβ/δ gene

(**A**) The splicing patterns used for generating mRNA initiated from the four different promoters. Exon 1A is spliced to exon 3; exon 1B is spliced to exon 2 which is spliced to exon 3; exon 1C is spliced to exon 2 which is spliced to exon 3; exon 1D is spliced to exon 3. (**B**) The 1B promoter harbours a CpG island. The most upstream transcription start point of the 1B promoter is situated at position 4940. Grey, CpG content; black, observed/expected occurrences of CpG. (**C**) Genomic Southern blot demonstrating the absence of PPARβ/δ pseudogenes.

Coupled *in vitro* transcription/translation and reverse transcriptase PCR

Coupled *in vitro* transcription and translation was performed with a TNT kit (Promega) as described by the manufacturer, using equimolar amounts of the following linearized templates which all contained the full-length coding region of the PPARβ/δ mRNA; -134 ($\sim$ 1A) containing the 1A 5′-untranslated region (UTR) from position -134; -548 ($\sim$ 1B1) containing the 1B1 5′-UTR from position -548; -284 ($\sim$ 1B4) containing the 1B 5′-UTR from position -284; -236 ($\sim$ 1C) containing the 1C 5′-UTR from position -236; -166 ($\sim$ 1D) containing the 1D 5′-UTR from position -166; and -31 ($\sim$ exon 3) containing exon 3-originating 5′-UTR from position -31. All constructs were cloned into pBluescript KS+. The products of the coupled transcription/translation were run on SDS/PAGE gels (10 %) in a Tris/tricine system with a resolving capacity down to approx. 2000 Da.

RNA was prepared from 20 μl of TNT product in Tris/tricine sample buffer by adding 200 μl of Gd-SCN buffer [4 M guanidine thiocyanate, 0.1 % antifoam A (Sigma), 25 mM sodium citrate, 100 mM β-mercaptoethanol, 0.5 % sodium *N*-lauroyl sarcosine, pH 7.0] and 10 μl of 2 M sodium acetate, pH 4.0. After vigorous vortexing, the RNA was extracted with 100 μl of phenol and 20 μl of chloroform. The aqueous phase was isolated, the RNA prepared with RNAtack resin (Biotecx) as described by the manufacturer and eluted in 40 μl of water. Isolated RNA (5 μl) was subjected to digestion with 5 units of RNase-free DNase I (Boehringer) in 1 mM $MgCl_2$ and reverse transcription was performed on 1.5 μl of RNA by M-MLV reverse transcriptase using the PPARβ/δ-specific primer 5′-TTCTAGAGCCCGCAGAATGG-3′. PCR was performed (20 and 25 cycles) with the primers 5′-GGCCATGGGTGACGGAGC-3′ and 5′-GATCTTGCAGATCCGATCGC-3′ and the products run on an agarose gel.

RESULTS AND DISCUSSION

The structure of the gene encoding murine PPARβ/δ

To isolate the murine PPARβ/δ gene, a λ genomic library was screened with a probe containing the entire open reading frame of the mouse mRNA encoding PPARβ/δ [23,24]. Two different

Table 1 Sequences of the intron/exon boundaries of the mouse gene encoding PPARβ/δ

The non-coding exon 1A is spliced to exon 3, the non-coding exons 1B and 1C are spliced to the non-coding exon 2 and the non-coding exon 1D is spliced to exon 3. Nucleotides in exons are indicated in upper case whereas the flanking nucleotides are in lower case. Numbers shown in parentheses are the exon sizes. Intron sizes are also given.

	Intron			
Exon	Donor	Size	Acceptor	Exon
1a (68 bp)	CCCTGgtgagtagag	25.6 kb	ccctgcccagGCAGT	3
1b (384 bp)	GACAGgtccgtgcgg	4.1 kb	caccttacagTGCTG	2
1c (72 bp)	TTCAGgcatgtgggt	380 bp	caccttacagTGCTG	2
2 (84 bp)	TAAAGgtaagcccgc	10.2 kb	ccctgcccagGCAGT	3
1d (47 bp)	CTGAGgtaagcaggg	6.5 kb	ccctgcccagGCAGT	3
3 (207 bp)	TGCAGgtatggaggg	8.8 kb	gtcttcacagACCTC	4
4 (155 bp)	GCAAGgtacagatgg	1592 bp	ggtctcgcagGGCTT	5
5 (139 bp)	CAACGgtgagggcgc	1053 bp	ccctatgcagCTATC	6
6 (203 bp)	ACGCAgtgagtgtca	105 bp	tggttttcagCCCTT	7
7 (451 bp)	TGGAGgtgaggggc	568 bp	gtccccacagACCGG	8
8 (1867 bp)				

clones were isolated and sequenced partially. The sequencing and comparison with rat [28] and human [22] PPARβ/δ sequences revealed the presence of additional exons in the 5′-end of the PPARβ/δ gene. Therefore, a mouse P1 bacteriophage library was screened with the intron-specific primers 5′-CCTGCAGGCCTGGTCATAGC-3′ and 5′-GGACAGCTGCGTCCTGAG-3′. Three clones were obtained and tested for upstream exon(s) by PCR with the rat-specific primers 5′-GGATTCTGCGGAGCGTGCG-3′ and 5′-CTGTCAGTGAGCAGGAGCCTC-3′. One P1 clone was found positive and was selected for further characterization. In parallel, 5′-RACE was performed on RNA from mouse liver using primers specific for part of the coding sequence (exon 3) to identify the first exon(s) of the gene. The products were cloned and the 5′-ends analysed by sequencing. Unexpectedly, a number of differentially spliced products were found, the most prevalent of which included or were initiated in exon 1B (Figure 1 and results not shown).

A library of subclones was generated from the P1 clone by cloning partially *Sau*3AI-digested P1 DNA into pBluescript KS+. Screening with specific oligonucleotides identified subclones harbouring exons 1B, 1C and 2. Subclones harbouring

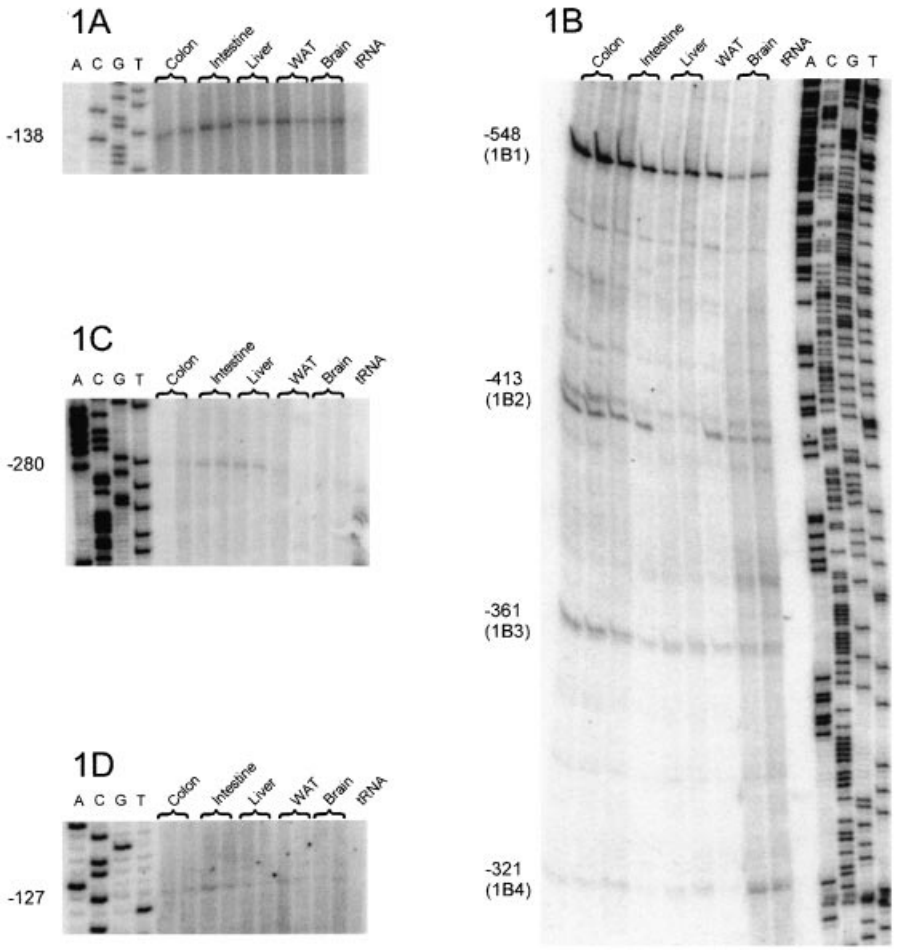

Figure 2 Determination of transcription initiation sites of the mouse PPARβ/δ 1A, 1B, 1C and 1D promoters

Primer extensions were performed using primers specific for exons 1A, 1B, 1C and 1D. Numbering is relative to the AUG start codon in the spliced mRNAs. Size standards were prepared by thermo-sequencing plasmid templates using the same primers. WAT, white adipose tissue.

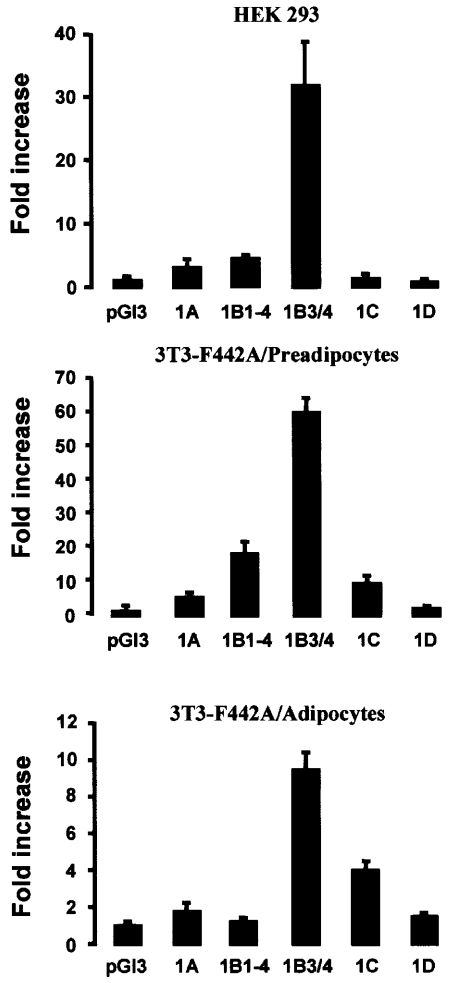

Figure 3 Promoter activity of the 1A, 1B, 1C and 1D promoters

The activities of the PPARβ/δ promoters in HEK-293 cells, 3T3-F442A preadipocytes and 3T3-F442A adipocytes (day 6) were determined after transient transfection with various PPARβ/δ promoter fragments harbouring $\approx$ 4 kb (1A), 222 bp (1B3/4), 834 bp (1B1-4), 2599 (1C) and $\approx$ 2.5 kb (1D) of the promoter regions cloned into pGL3 Basic. Promoter activity was determined relative to β-galactosidase activity as described, and normalized to the activity of pGL3 Basic. Results are shown as the means $\pm$ S.E.M. from three experiments.

exons 1A and 1D were generated by long-range PCR using primers designed from the cDNA sequences.

Thus the gene encoding PPARβ/δ spans approx. 41 kb (Table 1 and Figure 1A) and, in analogy with the genes encoding PPARα [21] and PPARγ [20,29], it comprises six exons in the 3′-end common to all transcripts. Tissue-dependent promoter usage (see below) and differential splicing yielded a variety of transcripts comprising one or two of the five exons present in the 5′-end of the mouse PPARβ/δ gene spliced to the common six exons located in the 3′-end of the gene. However, in contrast to PPARγ, neither of the mRNAs have the potential to encode variant PPARβ/δ isoforms. Interestingly, exon 2 is skipped when the first exon of the primary transcript is exon 1A. The most abundant mRNA has a predicted length of 3490 nt and exhibits high homology with the published sequence of the rat messenger [28], but contains only three CGG triplet repeats instead of the 14 repeats noted there. The human PPARβ/δ gene [22] had its transcription start point mapped to an exon with high homology to mouse exon 1B, contains no CGG triplet repeat and contains one additional non-coding exon placed between exons 1B and 2.

The splice donor/acceptors of the mouse PPARβ/δ gene conform to the splice consensus sequence GT/AG except the splice donor of exon 1C (Table 1). The homology between the 3′-UTRs of human and mouse PPARβ/δ is not high except for the last 159 nt, where a stretch of nucleotides exhibits 91 % identity and a conserved AATAAA polyadenylation signal is present.

Unlike the other PPARs, PPARβ/δ is ubiquitously expressed and is expressed early in embryonic development [24,30–32]. Exon 1B of the murine as well as exon 1 of the human gene [22] contain CpG islands [33] (Figure 1B and results not shown), a trait often associated with this family of housekeeping genes. Based on their expression in the germ line, most housekeeping genes have pseudogenes, but this is not the case for the murine PPARβ/δ gene (Figure 1C).

Determination of transcription start sites

Transcription start sites in the different promoters were determined by primer extension using primers complementary to sequences in the first exons (Figure 2). The transcription-initiation sites of exons 1A and 1C are situated 138 and 280 nt upstream of the AUG translational start codon in the processed mRNA, respectively; exon 1B has several transcription start sites although the site (1B1) situated at position -548 is preferred in all tissues examined. Exon 1D has a major initiation site at position -127, but also minor sites at positions -129, -298 and -329 (results not shown). Initiation site usage is differentially regulated, also within one promoter. One example is liver, where transcripts originating from transcription start point 1B2 are under-represented relative to those originating from transcription start points 1B1, 1B3 and 1B4 in other tissues (Figure 2). These results also corroborate the notion that multiple initiation sites are present in the 1B promoter and that the multiple bands observed by primer-extension analysis do not represent artifacts.

Analysis of promoter activity

The relative strength of the different promoters was compared by transient transfection in HEK-293 and 3T3-F442A cells using promoter constructs containing approx. 4 kb (1A), 2.6 kb (1C) or 2 kb (1D) of promoter sequences (Figure 3). A number of promoter constructs containing various fragments of the 1B promoter were also tested (Figures 3 and 1A). Whereas the 1B promoter (1B1-4, position -1089 to -256) seems to be the strongest in HEK-293 and 3T3-F442A preadipocytes, the 1C promoter seems to be more potent in 3T3-F442A adipocytes. Surprisingly, a minimal promoter containing only the 1B3 and 1B4 transcription-initiation sites (position -393 to -172) proved to have a very strong activity in all the cell types tested. This could indicate the presence of an element conferring transcriptional repression upstream of the 1B3 initiation sites or, alternatively, the presence of an element enhancing transcription between position -256 and -172, or a combination of both. Interestingly, the transcriptional repression or lack of activity of the possible positively acting downstream element is so pronounced in the differentiated adipocytes that the transcriptional activity of the 1B1-4 promoter is as low as that of the pGL3 vector without any inserted promoter elements in this cell type.

A search of the involved regions using the Matinspector program [34] revealed that a negative element between the 1B1 and 1B3 initiation sites could be a site binding the ubiquitously

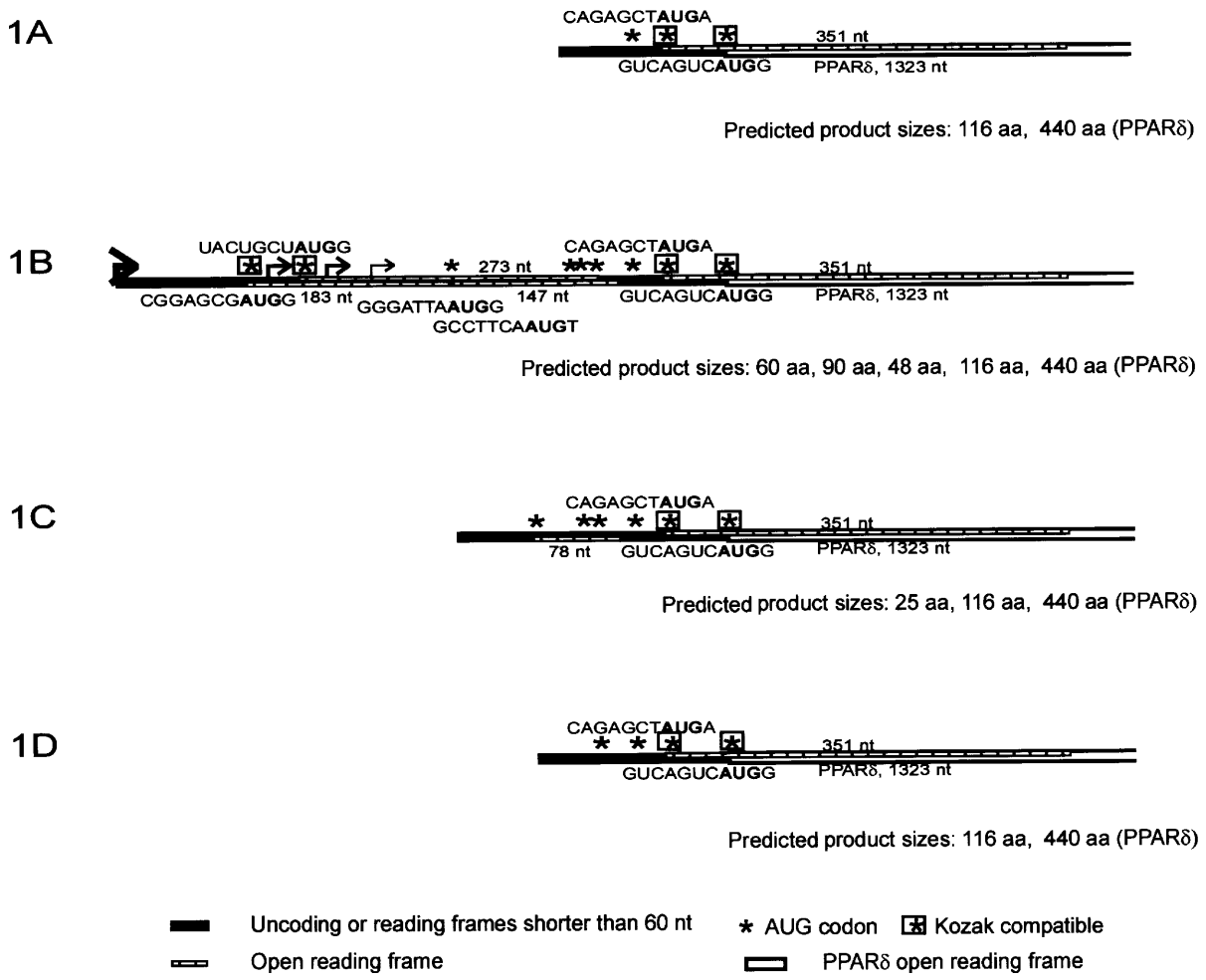

Figure 4 The 5′-ends of the processed PPARβ/δ mRNAs harbour good Kozak consensus sequences and long open reading frames

Shown is a schematic representation of transcripts initiated from the various PPARβ/δ promoters where the sizes of the leaders, the presence of AUG triplets and the sizes of possible open reading frames are indicated. AUG codons are marked by asterisks and AUG codons in good agreement with the Kozak consensus sequence are marked with boxed asterisks. Open reading frames larger than 60 nucleotides are shown as cross-hatched bars and the sizes of their predicted products indicated.

expressed bZip transcription factor TCF11 [35], which is able to repress transcription when bound to small musculoaponeurotic fibrosarcoma oncogene family (Maf) proteins, whereas two sites in the downstream promoter present only in the 1B3/1B4 construct could bind the ubiquitously expressed transcription factor activator protein 4 [36,37]. A more thorough promoter analysis will be necessary to fully characterize the promoters of the murine PPARβ/δ gene.

The activity of the 1C promoter is quite high in adipocytes *in vitro* whereas the 1C promoter seems to be only modestly used *in vivo* in fat (Figure 2). White adipose tissue consists of a number of cell types of which many are more transcriptionally active than the adipocytes. Thus the proportion of transcripts from the 1C promoter might be decreased in RNA isolated from adipose tissue due to the contribution of transcripts from cells other than adipocytes. Alternatively, the promoters used in the transfection assays may not include all elements used *in vivo* or the cell lines used may not reflect the *in vivo* situation when comparing the activities of the different promoters.

The data obtained from the 5′-RACE where the majority of clones obtained were 1B-derived indicated that the 1B promoter is predominantly used *in vivo* in the liver. Several lines of evidence indicate that 1B transcripts are the most abundant in a number of tissues, if not all. These data also include the primer extensions where the 1B primer extensions consistently yielded the strongest bands in a number of experiments (see Figure 2). Furthermore, only the 1B homologue was found in the studies concerning the human PPARβ/δ gene structure [22] and the rat PPARβ/δ cDNA [28].

Analysis of translational efficiency

An analysis of the 5′-UTRs resulting from transcription initiated from the different promoters revealed that especially the transcript initiated from the most upstream (major) 1B promoter contains a number of Kozak-compatible AUG codons [38] with associated open reading frames (Figure 4), whereas the shorter transcripts originating from the 1A, 1C and 1D promoters contain only a single Kozak-compatible AUG start codon. In vertebrates, translation is normally initiated from the first AUG codon with a strong Kozak consensus sequence whereas downstream AUG codons are not used.

Coupled transcription and translation in a rabbit reticulocyte system yielded markedly less product from a template transcribed

into the full-length 1B message [Figure 5, −548 (~ 1B1)] than transcripts generated from templates encoding 1A, 1C or 1D messages. Coupled transcription/translation of a template encoding an mRNA containing no upstream AUG codons [Figure 5, −31 (~ Exon 3)] gave rise to a significantly higher level of the translation product in comparison with the yield obtained from any of the exon 1A-, 1B-, 1C- or 1D-containing transcripts, emphasizing the role of the 5′-UTRs in attenuating translational efficiency.

We were unable to detect any products originating from alternative translation start points in the 1A, 1B, 1C or 1D messages in the rabbit reticulocyte lysate, as well as in a wheatgerm TNT system (results not shown), indicating that the upstream open reading frames are not translated or are only translated with very low efficiency, at least in the *in vitro* systems. Alternatively, any produced polypeptides may turn over very rapidly.

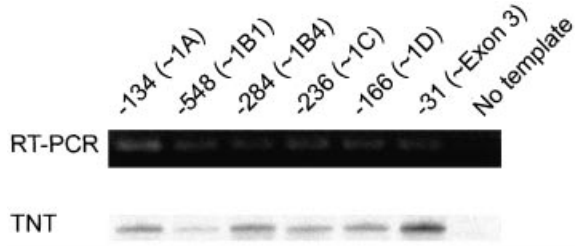

Figure 5 The 1B 5′-UTR inhibits translation

TNT, coupled *in vitro* transcription and translation with templates harbouring different UTRs from the gene encoding PPARβ/δ. RT-PCR, the amounts of transcripts were semi-quantitatively assessed by reverse transcriptase PCR. Various cycle numbers were tested and a representative gel is shown where the reactions were not saturated. The numberings correspond to the 5′-most position of the UTR relative to the A in the AUG start codon.

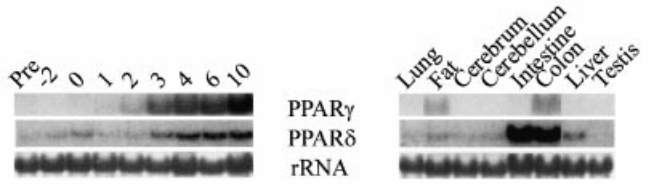

Figure 6 Tissue- and differentiation-specific expression of PPARβ/δ and PPARγ at the mRNA level

Northern blots were prepared using RNA from differentiating 3T3-L1 cells and from a number of mouse tissues. The results from probing with PPARβ/δ and PPARγ are shown. Equal loading/transfer was determined by Methylene Blue staining of rRNA on the blots. Numbers on the left indicate days in the differentiation process, where day −2 is the day of confluence of the cells.

Northern analysis (Figure 6) confirmed previous findings that PPARγ exhibits quite a tissue-restricted pattern of expression, whereas PPARβ/δ is ubiquitously expressed with the highest levels of expression in intestine, colon and appendix and only minute amounts of mRNA present in kidney and testis. Furthermore, expression of PPARβ/δ has been noted to be strongly induced during the differentiation of Ob1771 and 3T3-F442A preadipocytes [23], and is also induced, albeit to a lesser degree, in 3T3-L1 adipocyte differentiation (Figure 6).

Interestingly, expression of the PPARβ/δ protein (Figure 7) does not correlate with the mRNA levels (Figure 6). Thus there is a lack of induction of the PPARβ/δ protein during 3T3-L1 adipocyte differentiation (Figure 7, bottom row), and the expression of PPARβ/δ protein seems to be much lower in the colon and intestine than in a number of tissues (e.g. liver) where the mRNA levels are much lower. Taken together, these results suggest a marked regulation of PPARβ/δ at the level of alternative promoter usage and initiation of translation.

The regulation of translation by the usage of alternative promoters and/or alternative splicing and inhibition of translation by long 5′-UTRs containing AUG codons has been noted, especially for a number of genes encoding transcription factors and other regulatory proteins [39–42]. Thus the gene encoding the runt domain transcription factor AML1/RUNX1 is transcribed from two promoters; the UTR originating from transcription initiated at the proximal promoter contains two CpG islands able to form a stable stem-loop structure and 16 AUG codons. Translation of this mRNA is initiated through an IRES-mediated mechanism and is regulated in a cell- and differentiation-specific manner, whereas the mRNA initiated from the distal promoter is shorter, contains no GC-rich stretch and has translation initiated by a cap-dependent mechanism [40].

Since the structure of the gene encoding PPARβ/δ resembles the structure of the gene encoding AML1/RUNX1, we performed a series of experiments to investigate if the 5′-UTR of the 1B1-initiated transcript could mediate internal ribosome entry. A bicistronic construct was prepared and tested by transfection into NIH-3T3 fibroblasts. As expected, we found activity from both reporter genes, but an equivalent promoterless construct prepared as a control for internal promoter activity showed the same amount of downstream reporter gene activity, indicating that the activity of the second cistron in the first experiment was not necessarily the result of internal ribosome entry, but might also be due to transcriptional initiation directed by a promoter activity harboured in the 5′-UTR (results not shown and Figure 3).

A number of viral as well as cellular genes have, in analogy with the AML1/RUNX1 gene, been shown to be translationally initiated via an IRES-dependent mechanism. The cellular genes include the genes encoding platelet-derived growth

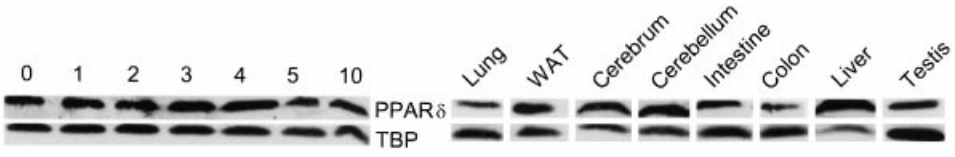

Figure 7 Tissue- and differentiation-specific expression of murine PPARβ/δ at the protein level

Protein was extracted from differentiating 3T3-L1 cells (left-hand panel) and from a number of mouse tissues (right-hand panel). They were subjected to Western blotting using a TATA-box-binding-protein-specific antibody (TBP) for normalization or a PPARβ/δ-specific antibody. The identity of the PPARβ/δ band was confirmed by its co-migration with PPARβ/δ obtained from PPARβ/δ-transfected NIH-3T3 cells. WAT, white adipose tissue. Numbers on the left indicate days in the differentiation process.

factor 2/*c-sis* [43], fibroblast growth factor 2 [44], a cardiac voltage-gated potassium channel [45], the max-binding protein (*mnt*) transcriptional repressor [46], c-Myc [47,48], apoptotic protease-activating factor (Apaf-1) [49,50], cationic amino acid transporter 1 (cat-1) [51], immunoglobulin heavy-chain-binding protein (BiP) [52], vascular endothelial growth factor [53], MYT2 [54], insulin-like growth factor II [55] and insulin-like growth factor I receptor [56]. In a number of cases where the evidence for a functioning IRES was obtained by transfecting bicistronic constructs into cells, the authors may have misinterpreted transcriptional activity or cryptic splice sites within the 5′-UTR (or postulated IRES). These events could lead to transcripts containing just the second cistron [57], which would then be translatable without internal ribosome entry.

In the PPARβ/δ transcripts initiated at the 1B1 promoter, the energetically most favourable folded secondary structure has a free energy of folding of -207.5 kcal/mol and will therefore pose a very difficult structure for a ribosome to traverse. It has been shown that initiation of translation of mRNAs with secondary structures of standard free energy values of folding up to approx. -30 kcal/mol is easily accomplished by the scanning mechanism, whereas the scanning 43 S preinitiation complex is stalled by more stable structures [58] and thus it seems unlikely that the translation of the longest PPARβ/δ transcript is initiated by the scanning mechanism, suggesting that the ribosomes enter the transcript internally. However, due to the presence of a cryptic promoter in the 5′-UTR further investigations are needed to determine if the translation of the most abundant PPARβ/δ transcript is accomplished by internal ribosome entry, by ribosome shunting or simply by the scanning mechanism.

An article describing the structure of the mouse PPARβ/δ gene [59] was published during the review process of the present article. This article describes the basic structure of the PPARβ/δ gene but only reports on the presence of eight exons (exons 1B and exons 2–8 in the present article) and hence the article does not describe the complexity of the promoter structure and the post-transcriptional regulation of the gene encoding PPARβ/δ.

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