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Characterization of the Mouse Peroxisome Proliferator-Activated Receptor δ Gene[1]

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Peroxisome proliferator-activated receptors (α, δ and γ) are ligand-activated transcription factors that are involved in multiple cellular responses. The PPARδ subtype is the least understood of all PPAR subtypes. PPARδ is activated by unsaturated fatty acids, PGI2, and by synthetic ligands. PPARδ-regulated genes have not been identified and the factors that control PPARδ expression are not known. The gene that encodes the mouse PPARδ gene is contained in >30 kb DNA sequence and organized in eight exons, six of which encode the PPARδ receptor. A PPARδ-luciferase reporter containing 694 bp 5′ upstream regulatory and 127 bp untranslated was introduced to primary brain cultures to begin a characterization of the DNA sequences that mediate transcriptional regulation of PPARδ. PPARδ-luciferase expression was 10 times higher in oligodendrocyte-containing mature cultures than in immature cultures, indicating that PPARδ may play a role during oligodendrocyte migration, proliferation, and/or maturation. © 2002 Elsevier Science

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Peroxisome proliferator-activated receptors (PPARs), are ligand-activated transcription factors that regulate gene expression and are involved in multiple cellular responses (1). The PPARα subtype, expressed mainly in the liver, heart, and kidney controls transcription of genes involved in lipid metabolism (2, 3) and transport (4), and mediates hepatic peroxisomal proliferation in mice (5). PPARγ2, a key regulator of adipogenesis (6), is activated by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15Δ-PGJ_2) (7), and is essential during early embryonic development (8). The PPARδ subtype, activated by fatty acids (9) and various xenobiotics (10), is expressed in various organs, including the brain. PPARδ has been studied in connection to adipogenesis (11), lipid metabolism (12), and blastocyst implantation (8). PPARδ-deficient mice exhibit demyelination in the corpus callosum, supporting the idea that this receptor may be required for proper oligodendrocyte formation and/or function (13).

In the vertebrate central nervous system, myelin, the lipid-rich biological membrane of oligodendrocytes, protects axons from injury and accelerates synaptic communication. Myelin contains proteins such as proteolipid protein (PLP) (14), myelin basic protein (MBP), 2′-3′-cyclic nucleotide 3′-phosphohydrolase (CNP), and protein zero (P_0) that are essential for its function (15). The various stages in the process of oligodendrocyte differentiation can be studied *in vitro* and can be distinguished on the basis of changes in cell morphology, mitotic potential and expression of stage-specific lipids and proteins (16, 17). Improved treatment and prevention of debilitating dismyelinating diseases necessitates a deeper understanding of the function and biosynthesis of myelin lipids and proteins. PPARδ is present in immature oligodendrocytes but not in astrocytes (18). Although PPARδ agonists such as bromopalmitate promote oligodendrocyte differentiation (19), the genes under PPARδ control have not been identified and the importance of this transcription factor in oligodendrocytes is not clear.

A productive approach toward understanding the physiological roles of PPARδ is the structural analysis of the PPARδ gene and an investigation of its regulation. The PPARδ gene is contained in at least 30 kilobases of DNA sequence and, like other nuclear receptor genes is organized in eight exons, six of which encode the PPARδ protein. The transcription initiation site was identified by primer extension and by rapid amplification of cDNA ends. A PPARδ-luciferase reporter that contains 694 bp 5′ upstream regulatory and 127 bp untranslated sequences was utilized to evaluate PPARδ gene expression in primary cultures isolated from neonatal rat telencephala, a source of immature oligodendrocyte progenitors (17). The levels of PPARδ-

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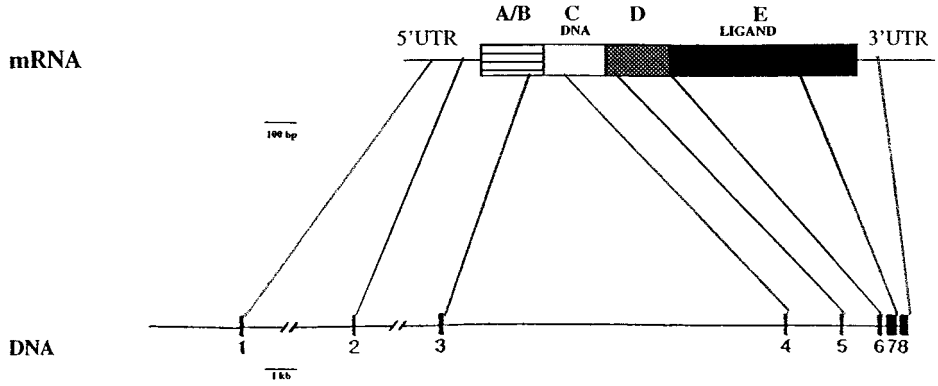

FIG. 1. Organization of the PPARδ gene. The domain organization of the mouse PPARδ mRNA is illustrated on top (A/B, transcription modulator domain; C, DNA binding domain; D, hinge region; E, ligand binding domain). The intron/exon organization of the mouse PPARδ gene is shown on the bottom. Exons are illustrated as boxes. The initiating ATG sequences are contained in exon 3.

reporter activity increase with time in culture, supporting a role of PPARδ during the progression through the oligodedroglial lineage.

MATERIALS AND METHODS

Isolation and mapping of mouse PPARδ genomic fragments. A PPARδ-P1 genomic clone (Genome Systems, Inc.) containing a >100,000 base pair insert was provided by Dr. Johan Auwerx. *Not*I, *Hin*dIII, *Eco*RI and *Xho*I fragments from this PPARδ-P1 clone were inserted by a "shotgun" procedure into the pBSIIKS cloning vector (Stratagene). We utilized oligonucleotide primers corresponding to N-terminal exons 5′ ATGACCAGGCCTGCAGCGGCCACGCAAGTG 3′ and 5′ TCCCAGAATTCCTCCCCTTCCTCCCTGCTGG 3′; to the DNA-binding domain 5′ TGCACCCCTCGCACGCGTGGACCCCG-TAGTG 3′ and 5′ TTCTTCCGCCGGACAATCCGCATGAAGCTCG 3′; and to the ligand binding domain 5′ ATCGAGACACTGTGGCAG-GCAGAGAAGGGCC 3′ and 5′ CTGCGGGCTCTAGAATTCCATCT-GCAGGTC 3′ to characterize overlapping subclones by Southern hybridization, polymerase chain reaction and DNA sequencing (20). The 5′ regulatory sequences and 5′ untranslated exons of the mouse PPARδ gene were isolated from a 129 SVJ mouse genomic library cloned into the bacteriophage Lambda FIX II (Stratagene). Radiolabeled DNA probes were prepared with the Rediprime kit (Pharmacia). The library was screened with the mouse PPARδ mRNA 5′ UTR isolated from mouse cDNA via rapid amplification of cDNA ends utilizing a Marathon kit (Clontech) employing the oligonucleotide 5′ GCTGGACTTGCCGGTGAGGATGCTCCGGGC 3′ as a gene specific primer. High stringency hybridization according to standard procedures (20) yielded positive phagemids that were characterized by Southern hybridization to the mouse PPARδ 5′ UTR. Appropriate fragments were subcloned into the pBSIIKS cloning vector (Stratagene) and sequenced. The Lasergene sequence analysis package (DNAStar) was utilized to merge individual sequences.

Identification of the mPPARδ transcription initiation site. Total RNA was isolated from mouse brain utilizing the RNeasy kit (Qiagen). The oligonucleotide 5′ AAACTTTCCCATTAATCCCCAGGTCC 3′ was radiolabeled by action of 10 units of T4 polynucleotide kinase in the presence of 30 μCi γ^{32}P-ATP according to standard procedures (20). Radiolabeled oligonucleotide (30 ng) was annealed to 2 μg mouse brain or yeast (control) RNA in 0.15 M KCl · 01 M Tris (pH 8.3) and 1 mM EDTA in 15 μl final volume for 12 h. RNA/oligonucleotide hybrids were templates for reverse transcription reactions conducted in 20 mM Tris (pH 8.3), 10 mM $MgCl_2$, 5 mM DTT,

TABLE I

PPARδ Exon/Intron Boundaries

Exon (bp)	Intron: Donor	Intron: Acceptor	Exon
1 (118)	gacagGTCCGTGCGG	CCTTACAGtgctg	2
2 (84)	taaagGTAAGCCCGCTTCGCAGA	CTCCCTGCCCAGgcagt	3
3 (207)	t gca gGTATGGATGGGG(>12 kb)	GTCTTCACAGac ctc	4
	A	*D L*	
4 (155)	gc aagGTACAGATGGACTG(~2 kb)	CCTTGGTCTCGCAGggc tt	5
	K	*G*	
5 (139)	c aac gGTGAGGGCGCCTGCGC(~1.3 kb)	CCCACCCCCTATGCAGct atc	6
	N	*A I*	
6 (203)	ac gcaGTGAGTGTCACTGGCC(103 bp)	GGGCCTGGTTTTCAGccc t	7
	A	*P*	
7 (451)	t gga gGTGAGGGGGCGGACC(50 bp)	CCCCACAGac cgg	8
	G	*D R*	
8 (>287)	tg taaggcc		
	Y *		

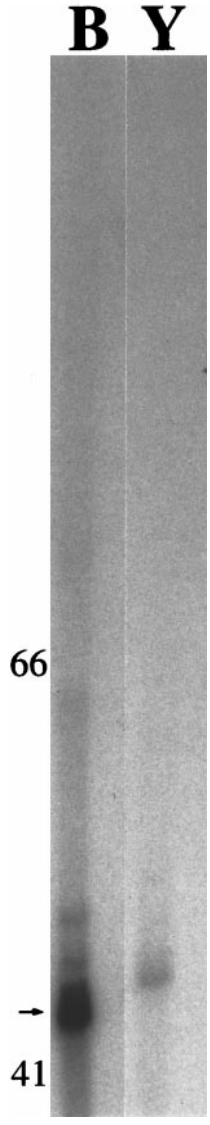

FIG. 2. Primer extension analysis. Autoradiograph of mouse brain RNA (B) or yeast RNA control (Y) primer extension products. The numbers on the left indicate polydeoxynucleotide length in base pairs.

150 μg/mL actinomycin D, 150 μM dNTPs and 5 units AMV reverse transcriptase for 1 h at 42°C. The samples were treated with 100 μg/ml RNase A and 10 mg/ml RNase T1 for 15 min at 37°C, extracted with phenol, precipitated with ethanol and resolved by electrophoresis in 9% acrylamide/7 M urea polyacrylamide gels. PBSII KS cloning plasmid (Stratagene) dideoxy sequencing reaction products were resolved in parallel lanes to determine the length of the primer extension products. Autoradiograms were developed after 48 h exposure at −80°C.

Cell culture and transfection. Primary cultures were obtained from 2-day-old rat telencephala according to a published procedure (21). After removal of the meninges, telencepha from 10 two-day-old rats was digested in 0.25% trypsin in DMEM (Life Technologies) for 30 min at 37°C. Cell clumps were gently triturated by passage through a 5-ml pipet to dissociate cells. Cells were collected by centrifugation and purified by filtration through a 4% bovine serum albumin solution. Cells were resuspended in 5% FPD (DMEM supplemented with 5% fetal bovine serum) and seeded in 35-mm dishes at 750,000 cells/dish. Cells were transfected on the 3rd or 10th day *in vitro* (DIV) and harvested 2 days later, on the 5th or 12th DIV.

The pGL3 basic plasmid (Promega) contains the firefly *(P. pyralis)* gene. To obtain the mPδ664 reporter construct, 694 bp 5′ flank and 127 bp UTR sequences were inserted in the pGL3 basic plasmid with the UTR sequences immediately upstream the luciferase gene. In the mPδ664rev control plasmid, the 694 bp 5′ flank and 127 bp UTR sequences are in the opposite orientation. The pRL-TK plasmid (Promega) contains the *Renilla (R. reniformis)* luciferase under the control of the herpes virus thymidine kinase promoter and was included as an internal control in all transfection DNA mixes. The Effectene reagent (Qiagen) was utilized to introduce DNA mixes into primary cultures according to manufacturer's specifications. Cells lysates were processed according to the Dual luciferase system (Promega) and analyzed for luciferase activity in a Turner luminometer. The relative light units (RLU) were calculated by dividing the firefly luciferase light units by the *Renilla* luciferase light units for all samples. Average RLUs (n = 3) for lysates obtained from cells transfected with the mPδ664 and mPδ664rev reporters were divided by the RLUs obtained from cells transfected with the pGL3 basic plasmid.

RESULTS AND DISCUSSION

The Structure of the Mouse PPARδ Gene

Long-range PCR results revealed that the coding exons of the mouse PPARδ gene were all present in a bacteriophage P1 (PPARδ-P1) genomic clone (data not shown). The 5′ UTR sequences absent in the PPARδ-P1 clone, were isolated from a bacteriophage Lambda FIX II mouse genomic library. The sequence information obtained from relevant subclones was compared to a published mouse PPAR cDNA sequence (22). Figure 1 illustrates the organization of the mouse PPARδ gene. The 282 bp of 5′ UTR of the mouse PPARδ mRNA are encoded by exons 1, 2, and 3. Exon 3 encodes the initiating methionine, a structural feature also present in the mouse PPARα (23) and PPARγ1 (24) paralog genes. Similarly to the human PPARδ ortholog gene (25), Intron 3 interrupts the 43rd codon after its first nucleotide. Exon 4 encodes the carboxyl end of the A/B modulator domain and the amino acids that form the first zinc finger of the DNA binding domain, ending on the lysine-encoding AAG codon that follows the codon codifying the last cysteine of the first zinc finger. Therefore intron 4, a type 0 intron, interrupts the exons that encode the two zinc fingers of the receptor's DNA binding domain. This feature is shared with the human PPARδ ortholog (25), the PPAR γ/γ1 paralog genes (24) and other receptors that fall within the thyroid hormone and retinoid receptor subfamilies (26, 27). In the mouse PPARα (23) gene, this intron begins one nucleotide earlier (23). Exon 5 encodes the second zinc finger and remaining amino acids of the DNA binding domain. Exon 6 encodes the hinge region separating the DNA binding domain from the ligand biding domain which is encoded by exons 7 and 8, a structural organization shared by all PPAR genes studied to date. Table I illustrates that all introns conform to the

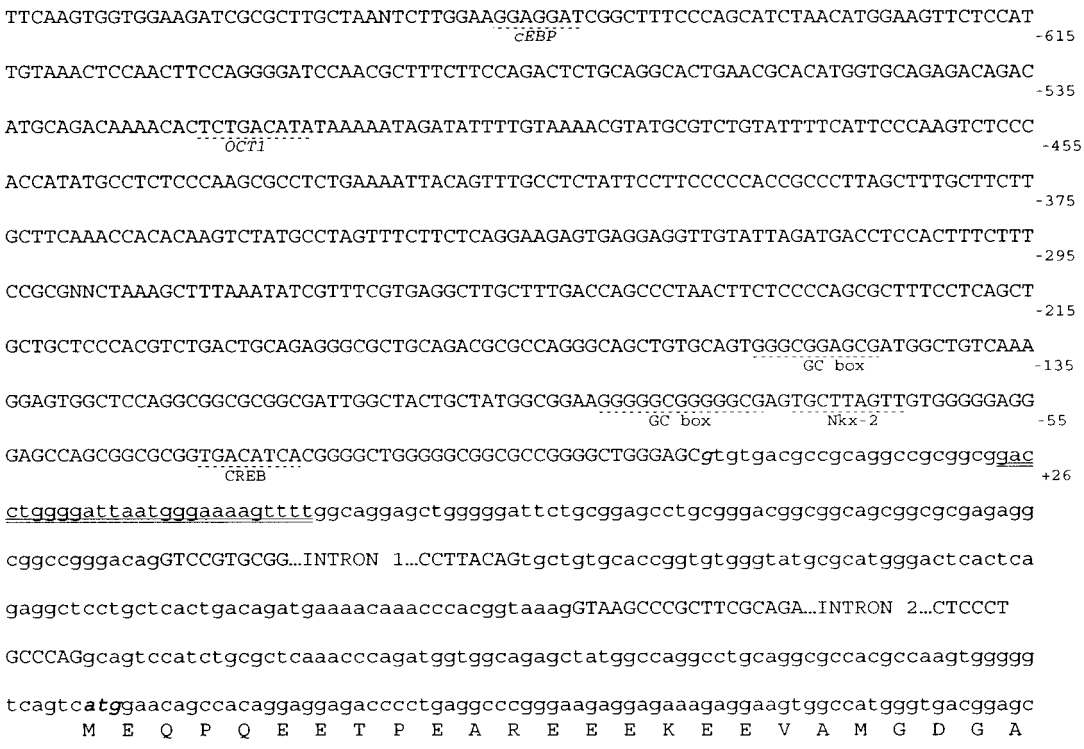

FIG. 3. The mouse PPARδ 5′exons and intron boundaries. The transcription initiation site and initiating 5′ ATG 3′ sequences are in bold italics with the predicted amino acid sequence of the first 25 amino acids under the codifying nucleotide sequences. The 5′ flank sequences and introns are capitalized. The sequence of the oligonucleotide utilized in the primer extension reactions is double underlined. Potential *trans*-acting factor recognition sites are underlined with dashed lines and labeled. Numbers on the right of figure are the nucleotide position relative to the transcription initiation site.

GT/AG splicing rule and that introns 3, 5 and 7 are type I introns whereas introns 4 and 6 are type 0 introns.

Analysis of the Mouse PPARδ 5′ Region

Two complementary approaches revealed the boundary between the regulatory and the structural regions of the mouse PPARδ gene. The 5′ UTR was amplified by rapid amplification of cDNA ends (RACE) utilizing the oligonucleotide 5′ GCTGGACTTGCCGGTGAGGATGCTCCGGGC 3′, which anneals in the DNA sequences encoding the hinge region. The 897 bp amplification product obtained in these reactions contains 282 bp 5′ UTR and 615 bp coding sequence (data not shown). Primer extension analysis demonstrated that the PCR product obtained in RACE reactions is representative of the predominant PPARδ mRNA form in the brain. As shown in Fig. 2, a 49 bp product was obtained in reverse transcription reactions utilizing mouse RNA and the oligonucleotide 5′ AAACTTTCCCATTAATCCCCAGGTCC 3′, verifying that the G found 282 bp upstream the initiating AUG is the predominant transcription initiation site. As shown in Fig. 3, two introns interrupt the 5′ UTR of the mouse PPARδ gene, a common structural characteristic of nuclear receptor genes present in genes that encode receptors that differ from PPARs such as the oxysterol receptors (LXRα and LXRβ) (28) and the retinoic acid receptors (RARs) (29).

The transcriptional regulation of the mouse PPARδ gene is not known. A database search of potential binding sites for transcription factors (30) on the 5′ region of the PPARδ revealed information that will guide future studies. Nuclear receptor genes do not employ a TATA box to mediate basal rates of transcription (31). Inspection of the nucleotide sequence of the 5′ flank of the mouse PPARδ gene revealed 5 GC boxes, two of which are shown in Fig. 3. An imperfect cAMP responsive element binding protein (CREB) recognition site exists approximately 30 bp upstream the transcription initiation site. It will be interesting to determine if this CRE is active and how it affects PPARδ expression. Recognition sites for transcription factors thought to be involved in cell proliferation and development such as Oct-1 (32) and Nkx-2 (33, 34) were also found. Deletion analysis may indicate if these sites are functionally relevant for expression of PPARδ mRNA.

The 5′ regulatory sequences of the PPARδ gene were further studied in primary brain cultures. Initially, these cultures consist mostly of neurons, astrocytes

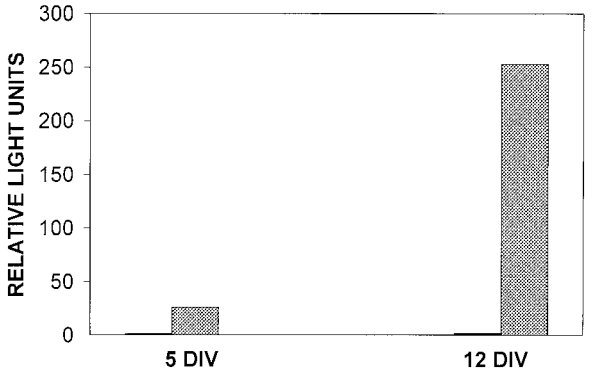

FIG. 4. PPAR-reporter expression in mixed brain cultures. Cells were transfected after 3 or 10 days in culture with either mPδ664rev (black bars) or mPδ664 (stippled bars) plasmids and harvested after 5 DIV or 12 DIV.

and oligodendrocyte progenitors. The culture conditions used do not support neuronal survival and therefore after 5 DIV the majority of cells in the cultures are astrocytes and oligodendrocytes at initial stages of differentiation. Cultures are not supplemented with growth factors that affect oligodendrocyte differentiation (35, 36) and therefore by the 12th DIV most oligodendrocytes begin to express differentiation markers such as myelin basic protein. Published data indicate that in mixed cultures oligodendrocytes, but not astrocytes, express PPARδ mRNA and that PPARδ activators promote oligodendrocyte differentiation (18). Brain cell primary cultures are therefore a physiologically relevant system to study the mouse PPARδ 5′ regulatory sequences. Immature cells were transfected after 3 DIV, and harvested 2 days later. Older cultures containing oligodendrocytes committed to the oligodendroglial lineage were transfected at 10 DIV and harvested two days later, at 15 DIV. Cells were harvested and lysed, and luciferase activity was measured. As shown in Fig. 4, the DNA sequences contained in the mPδ664rev control plasmid failed to promote firefly luciferase activity at 5 or 12 DIV in mPδ664rev-transfected cells. In contrast, cells transfected with the mPδ664 plasmid, which contains PPARδ genomic sequences correctly oriented relative to the luciferase gene, express 25-fold higher levels of luciferase than cells transfected with pGL3 basic at 5 DIV and 253-fold higher levels of luciferase at 12 DIV. Therefore, cells transfected and harvested after 12 DIV display a 10-fold increase in mPδ664-reporter activity relative to more immature cells harvested at 5 DIV. It is likely that astrocytes and other contaminating cells may contribute to the luciferase levels measured and therefore it is important to study the mPδ664 reporter in pure and developmentally synchronized oligodendrocyte cultures.

CONCLUSIONS

PPARδ-deficient mice were created by deleting the exons that encode the receptor's ligand binding domain without a complete characterization of the PPARδ gene (13). The observed phenotype included subtle myelin content in the corpus callosum, a difficult result to interpret because PPARδ-null mice express PPAR mRNA in brain. It would be interesting to determine if deletion of additional exons would yield a different phenotype. The elucidation of PPARδ-regulated genes and a mechanistic understanding of the factors that influence PPAR expression will further our understanding of this receptor.

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