PPARγ3 mRNA: a distinct PPARγ mRNA subtype transcribed from an independent promoter

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Abstract PPARγ is a member of the peroxisome proliferator activated receptors (PPAR) subfamily of nuclear receptors. So far two PPARγ isoforms, PPARγ1 and PPARγ2, were known in mammals. We describe the structure of a novel human PPARγ subtype, PPARγ3. The PPARγ3 mRNA is transcribed from a novel promoter localized 5′ of exon A2. PPARγ3 mRNA expression was restricted to adipose tissue and large intestine. Similar to human PPARγ1 and -2, PPARγ3 is activated by thiazolidinediones and prostaglandin J derivatives and binds with high affinity to a PPRE.

Key words: Adipocyte differentiation; Nuclear hormone receptor; Insulin sensitivity

1. Introduction

The peroxisome proliferator activated receptors or PPARs are a subfamily of the nuclear hormone receptor gene superfamily. So far three distinct PPARs, α, δ, and γ, each encoded by a separate gene and with a specific tissue distribution have been described. Two important findings recently underscored the importance of the PPARγ transcription factor. First, PPARγ has been identified as one of the key factors controlling adipocyte differentiation and function [1,2], hinting to a potential role for it in metabolic disorders linked to abnormal adipose tissue physiology, such as obesity and non-insulin dependent diabetes mellitus (NIDDM); second, the recent identification of prostaglandin J2 derivatives and thiazolidinediones as, respectively, natural and synthetic PPARγ ligand [3–7]. Thiazolidinediones are a new group of anti-diabetic drugs which improve insulin resistance (reviewed in [8,9]). These observations have generated a major interest in the role of this PPAR subtype in normal and abnormal adipocyte function in man.

We recently determined the gene structure of the human PPARγ gene and started studying its regulation and expression. When analyzing the expression of human PPARγ, a novel subtype, PPARγ3, was identified. The expression of this PPARγ3 mRNA was directed by an independent promoter and was confined to adipose tissue and colon epithelium. The PPARγ3 mRNA gave rise to a protein indistinguishable from PPARγ1. PPARγ3 expression was, like PPARγ2, restricted to colon and adipose tissue in man. The identification of a third independent promoter suggests a complex regulation of the PPARγ gene expression and allows a more accurate regulation of this transcription factor.

2. Materials and methods

2.1. Materials and oligonucleotides

The oligonucleotides used for various experiments in this manuscript are listed in Table 1. BRL 49 653 was a kind gift of Dr. A. Nazdan of Ligand Pharmaceuticals. All chemicals, except if stated otherwise, were purchased from Sigma (St. Louis, MO, USA).

2.2. RNA isolation, primer extension, and RNase protection assays

Total cellular RNA was prepared as described previously [10]. For primer extension, the oligonucleotide LF-60 was ^{32}P-labelled with T4-polynucleotide kinase (Amersham, Courtaboeuf, France) to a specific activity of 10^7 dpm/50 ng and purified by gel electrophoresis. Primer extension analysis was performed using 50 μg of total RNA and 10^5 dpm of radiolabelled oligonucleotide according to a standard protocol utilizing a mixture of 1.25 U AMV reverse transcriptase (Life Technologies, Paisley, UK) and 100 U MMLV reverse transcriptase (Life Technologies, Paisley, UK). A sequencing reaction was used as molecular mass standard to map the 5′ end of the extension products.

The absolute mRNA concentration of the differentially spliced PPARγ variants was measured by RNase protection assay exactly as described [11]. The full length PPARγ2 coding region plus 33 bp of the 5′-UTR was amplified from human adipose tissue RNA by RT-PCR with the primer pair LF-3/LF-36 and was inserted in inverted orientation (3′ to 5′ in front of the T7 promoter) into the *Eco*RI site of the expression vector pSG5 (Stratagene, La Jolla, CA, USA). The resulting plasmid pSG5-hPPARγ2-inv was digested with *Eco*RV and religated, yielding the vector pSG5-hPPARγ2-RPA, which was used as a template for the synthesis of the anti-sense RNA probe, to measure the amounts of PPARγ2 relative to PPARγ1 and 3 mRNA. For the specific analysis of the PPARγ3 mRNA relative to PPARγ1, another probe template was constructed by RT-PCR from human adipose tissue RNA with the primer pair LF-44 (which binds to the sense strand in exon A1) and LF-21 (which binds to the antisense strand in exon 2). The amplified fragment, which contains part of exon A1, the full length exon A2, exon 1, and part of exon 2, was inserted into the *Eco*RV site of pBluescript SK$^+$ to generate the plasmid pBSγ3-RPA. For the analysis of mouse PPARγ RNA, mPPARγ cDNA was amplified, using the same strategy and oligonucleotides described above to create the vector pSG5-mPPARγ2-inv. This plasmid was digested by *Eco*RI and religated resulting in the plasmid pSG5-mPPARγ-RPA. The in vitro synthesized probe contains part of exons 4 and 5 of the mPPARγ gene.

2.3. Tissue biopsies and cell culture

Omental adipose tissue, small and large intestine, kidney, muscle, and liver biopsies were obtained from non-obese adult subjects undergoing elective surgery or endoscopy. All subjects had fasted overnight before surgery (between 8.00 p.m. and 10.00 a.m.) and received intravenous saline infusion. They had given informed consent, and the project was approved by the ethics committee of the University of Lille. All tissue was immediately frozen in liquid nitrogen until RNA preparation. Standard cell culture conditions were used to maintain 3T3-L1 (ATCC), CV-1 (a kind gift from Dr. R. Evans), THP-1 (ATCC), HeLa (ATCC), CaCo2 (ATCC) and HepG2 cells (ATCC). In certain experiments superconfluent differentiated CaCo2 cells were

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Table 1
Oligonucleotides used in this study listed from 5′ to 3′

LF60	cgttaaaggctgactctcgtttga
LF65	cgttaaaggctgactctcgtttga
LF36	gtcaccgaattctagtacaagtccttgtagatctcc
LF3	gtgaattacagcaaacccctattc
LF44	gtcggcctcgaggacaccggagag
LF63	gtcacatgaatgacgatacctc
LF68	tcatgtaggtaagactgtgtagaa
LF102	ctagcgtcattcatgtgacataaa
AII-J	gatccttcaacctttaccctggtaga

used. BRL 49 653 (in DMSO) was added to the medium at the concentrations and times indicated. Control cells received vehicle only.

2.4. Isolation of the human PPARγ3 cDNA

In order to isolate the full length PPARγ3 cDNA, RT-PCR was performed using the primer pair LF-65 (binding at the beginning of exon A2 of the human PPARγ gene) and LF-36 (binding at the stop codon) and human adipose tissue RNA as template. The resulting amplified fragment was cloned blunt into the *Eco*RV site of pBluescript SK$^+$ (Stratagene, La Jolla, CA, USA) and sequenced on an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA). The fragment containing the full length PPARγ3 cDNA was then cloned into vector pcDNA3 (Invitrogen, Leek, The Netherlands) resulting in the expression vector pcDNA3-hPPARγ3 which was used in both transfection and gel-shift experiments.

2.5. Analysis of promoter activity and transactivation assays

The PAC clone P-8856 [12], containing the full length PPARγ gene, was sequenced with the oligonucleotides LF-60 and LF-63 pointing upstream of exon A2. A 800-bp fragment of the PAC clone 8856 was isolated by PCR using the amplimers LF-60 (binding to the antisense strand in exon A2) and LF-68 (binding sense at position −800 of the PPARγ3 promoter). This PCR fragment was sequenced, inserted into the *Eco*RV site of pBluescript SK$^+$ (Stratagene, La Jolla, CA, USA), and after *Spe*I and *Kpn*I restriction subcloned into pGL3 (Promega, Madison, WI, USA), creating the reporter vector pGL3γ3p800. Transfections, luciferase and β-galactosidase assays were generally performed as described previously [13].

2.6. Electrophoretic mobility shift assays (EMSA) and oligonucleotide sequences

hPPARγ3 and mRXRα [14] proteins were synthesized in vitro in rabbit reticulocyte lysate (Promega, Madison, WI, USA). Molecular weights and quality of the in vitro translated proteins were verified by SDS-PAGE. hPPARγ (2 μl) and/or mRXRα (2 μl) were incubated for 15 min on ice in a total volume of 20 μl with 1 ng of T4-PNK end-labelled AII-J-PPRE double-stranded oligonucleotide probe, 2.5 μg poly(dI:dC) and 1 μg herring sperm DNA in binding buffer (10 mM Tris-HCl (pH 7.9), 40 mM KCl, 10% glycerol, 0.05% Nonidet P-40 and 1 mM DTT). For competition experiments, increasing amounts (from 10- to 200-fold molar excess) of cold oligonucleotide (AII-J-PPRE [15]: 5′-GATCCTTCAACCTTTACCCTGGTAGA-3′) were included just before adding labelled probe. DNA-protein complexes were separated by electrophoresis on a 4% polyacrylamide gel in 0.25× TBE buffer at 4°C [16].

3. Results

We have previously determined the sequence of the exon-intron boundaries of the human PPARγ gene [12]. A more detailed analysis of the 5′ sequence boundaries of exon A2

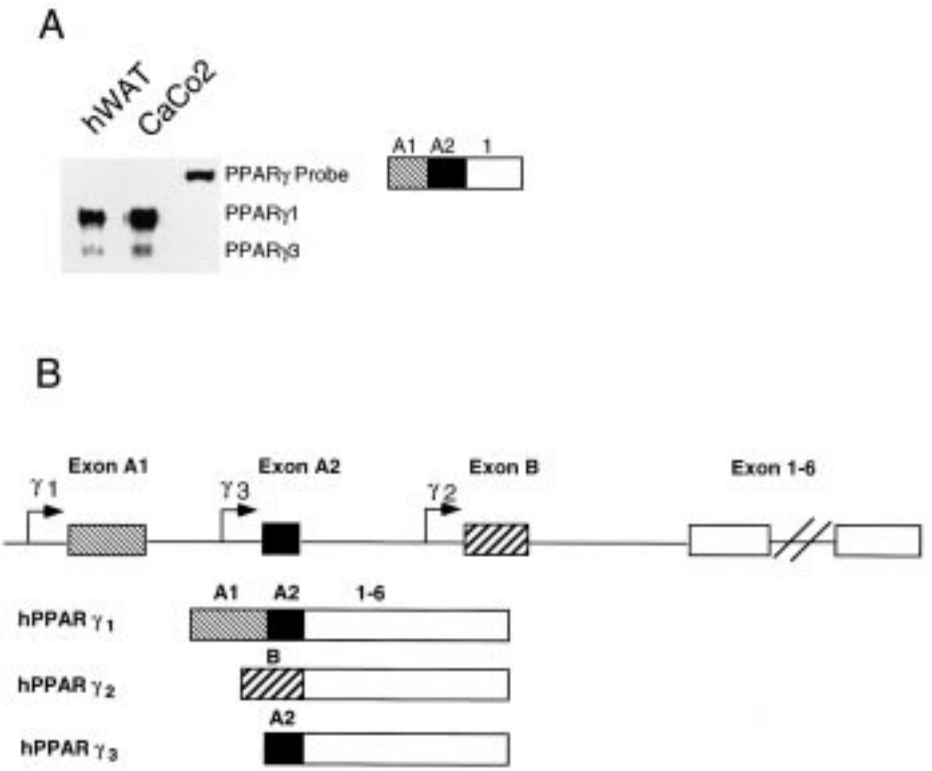

Fig. 1. RNase protection assay demonstrating the presence of a third PPARγ form (A) and schematic representation of the genomic structure of the human PPARγ gene (B). A: RNase protection assay showing the presence of a band corresponding to an mRNA message containing only exon A2 in both human omental white adipose tissue and in the human adenocarcinoma cell CaCo2. A scheme of the probe is indicated at the right side of the autoradiograph. The bands corresponding to the undigested probe as well as the protected fragments corresponding to PPARγ1 and PPARγ3 are indicated. B: A scheme of the genomic structure of the 5′ end of the human PPARγ gene. Exons 1–6 are shared by all three subtypes. PPARγ1 contains in addition the untranslated exons A1 and A2, PPARγ2 contains exon B, which is translated, and PPARγ3 contains only the untranslated exon A2.

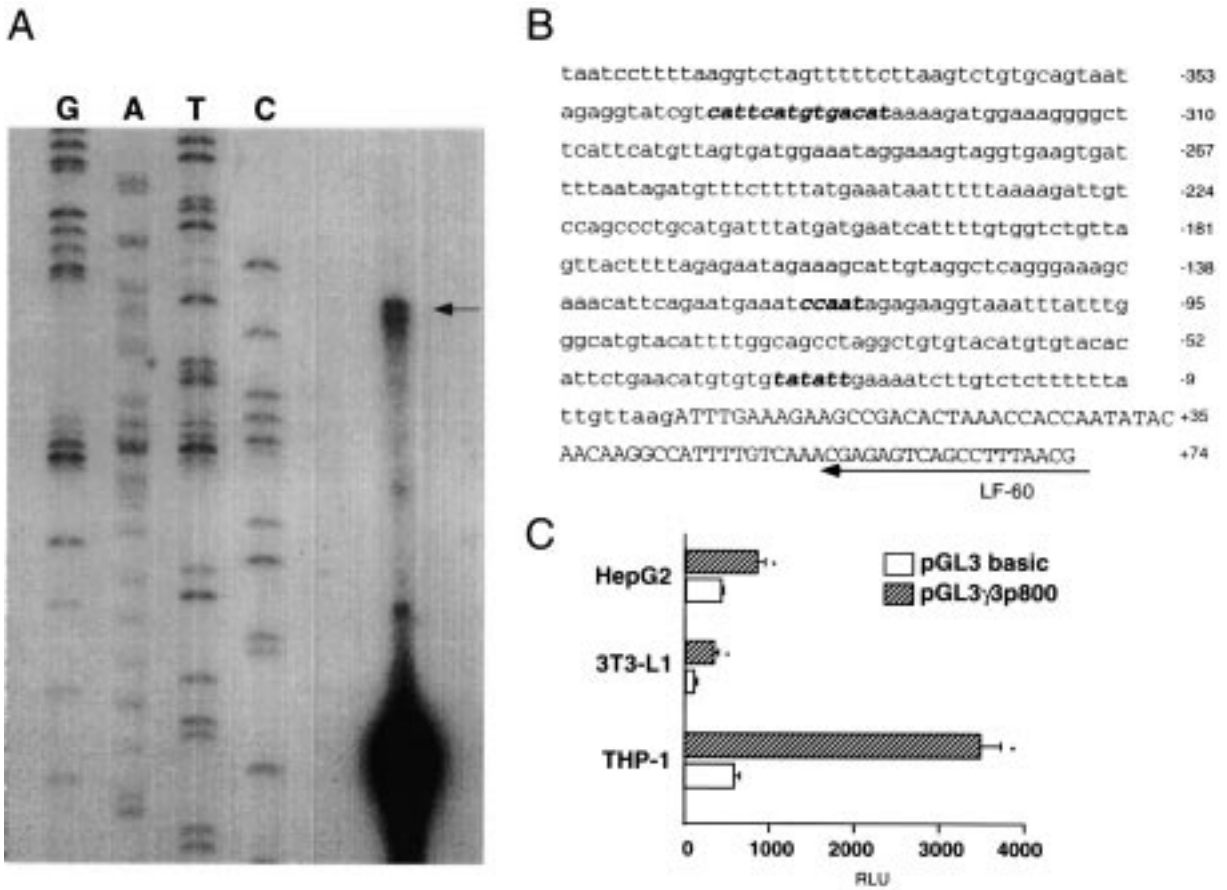

Fig. 2. Determination of the transcription initiation site of PPARγ3, sequence of proximal 5′ regulatory region, and analysis of PPARγ3 promoter activity. A: Primer extension of human white adipose tissue RNA with the PPARγ specific primer LF-60 was performed as described in Section 2. The major extension product is indicated by an arrow. Size standards, on the left, consist of a sequencing reaction. B: Sequence corresponding to the 5′-UTR of the PPARγ3 mRNA. Sequence motifs are indicated in bold (CCAAT box, TATA box). The oligonucleotide LF-60 used for primer extension is indicated by the arrow. C: Relative activity of the PPARγ 3 promoter. Normalized luciferase activity of the pGL3-γ3p800 reporter construct, containing 800 bp of PPARγ3 promoter, were determined after transient transfections in 3T3-L1, HepG2, and THP-1 cells as described in Section 2. Results represent the mean ± the standard deviation of three independent experiments. Asterisks indicate statistically significant differences by Student's *t*-test.

indicated the presence of some potential regulatory elements such as a TATA and CCAAT box motif. These data suggested the presence of a third promoter in the PPARγ gene, which could lead to the production of an alternative PPARγ mRNA species containing only exon A2 in its 5′-UTR. In order to assess this possibility, an RNase protection assay was performed using a radiolabelled probe containing both the A1 and A2 untranslated exons plus a sequence common for all PPARγ mRNAs, i.e. exon 1. In the presence of a PPARγ mRNA species containing exclusively exon A2, this probe would yield a protected fragment which is 42 bp shorter than the fragment present in PPARγ1, which contains both exons A1 and A2. These 42 bp correspond to the part constituted by sequences from exon A1 present in the probe (Fig. 1A). Consistent with this idea, RNase protection assays of RNA from adipose tissue and colonic CaCo2 cells, two tissues which are known to express high levels of PPARγ, showed an additional RNA species, different from PPARγ1 and -2. The size of this new mRNA species corresponds to an RNA which contains only exon A2 (Fig. 1A). Based on this information, the structure of the 5′ end of the human PPARγ gene corresponds to the scheme represented in Fig. 1B.

We next performed primer extension experiments on human adipose tissue RNA to identify the 5′ end of this novel PPARγ cDNA. One major primer extension product of 37 bp was observed with the primer LF-60 (Fig. 2A). The length of the extended product indicates that this new mRNA begins at the initiation of exon A2. These data unequivocally demonstrate the presence of an additional PPARγ transcription initiation site giving rise to a new PPARγ mRNA which we designated as PPARγ3. The PPARγ3 mRNA will, however, be translated into a protein which is indistinguishable from PPARγ1 since there is no translation initiation site in exon A2 (Fig. 2B). The region 5′ to the transcription initiation site of the PPARγ3 mRNA, which corresponds to the proximal PPARγ3 promoter, was sequenced (Fig. 2B). Several consensus sequence elements were identified. A TATA-like element was found at −34 relative to the transcription initiation site. Sequence analysis furthermore identified a potential CAAT-like consensus C/EBP binding site at −118 as well as a potential E-box binding site for the transcription factor ADD-1/SREBP-1 at position −342 (Fig. 2B).

To evaluate the activity and tissue specificity of this promoter we cloned an 800-bp fragment, located immediately upstream of the transcription initiation site of PPARγ3 into the luciferase reporter vector pGL3 basic. The resulting plas-

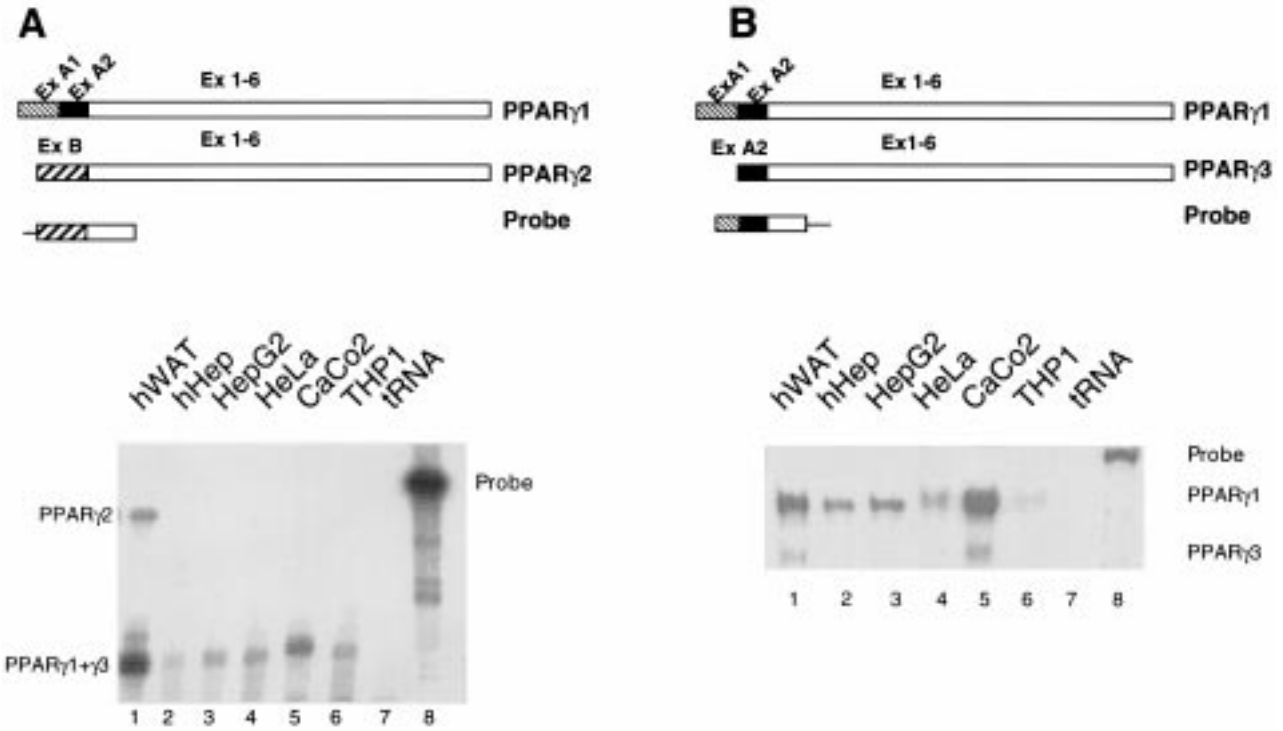

Fig. 3. Analysis of the expression of the different PPARγ isoforms in human cells and tissues. A: RNase protection assay comparing the expression of PPARγ2 relative to PPARγ1 and PPARγ3 mRNA in the indicated tissues and cell lines. The RNA from the CaCo2 cells was obtained from differentiated cells, whereas that of THP-1 cells was from undifferentiated cells. The structure of the mRNAs as well as the probe used in the assay is indicated in the top of the panel. B: RNase protection assay comparing the expression of PPARγ3 relative to PPARγ1 and mRNA in the indicated tissues and cell lines (same as in panel A). The structure of the mRNAs as well as the probe used in the assay is indicated in the top of the panel.

mid pGL3γ3p800 was transfected into the mouse 3T3-L1 and human HepG2 and THP-1 cell lines (Fig. 2C). Transfection efficiency was monitored by evaluation of the activity of a β-galactosidase control vector. Relative to the promoterless parent vector, the PPARγ3 promoter stimulated the luciferase expression more than two-fold in HepG2 cells and in undifferentiated 3T3-L1 cells (Fig. 2C). The activity of the PPARγ3 promoter was in the same range as the activity observed for the PPARγ1 and -2 promoter in these cells (data not shown and [12]). Interestingly, in THP-1 cells, a myeloid cell line, expression of the PPARγ3 promoter was more than six-fold higher than that of an promoterless control construct (Fig. 2C).

In order to study the relative expression of the three PPARγ subtypes in different tissues we employed RNase protection assays. Due to the complexity of the PPARγ gene we could not quantitate in the same assay the relative levels of expression of the three PPARγ mRNA forms. Therefore we performed two different assays (see Section 2 for the description of the probes). The first RNase protection assay was designed to determine the amount of PPARγ2 mRNA relative to PPARγ1 and PPARγ3 mRNAs (Fig. 3A), whereas with the second RNase protection assay we determined the amount of PPARγ3 mRNA relative to that of PPARγ1 (Fig. 3B). The expression of PPARγ3 mRNA is restricted to adipose tissue and differentiated CaCo2 cells (Fig. 3B, lanes 1 and 5). With the exception of human white adipose tissue, none of the tissues or cell lines analyzed contained substantial amounts of PPARγ2 mRNA (Fig. 3A). In contrast to the tissue restricted expression of the PPARγ2 and -3 mRNAs, PPARγ1 mRNA is more widely expressed and is detected in adipose tissue, human hepatocytes, and the cell lines HepG2, CaCo2, and HeLa (Fig. 3A,B).

Although sequence analysis suggested that the PPARγ3 protein should be identical to the protein product arising from the PPARγ1 mRNA, we verified experimentally whether the protein made from PPARγ3 expression vector has similar characteristics. To analyze whether PPARγ3 could bind to a PPRE, classically composed of direct repeats spaced by one intervening nucleotide (DR-1), electrophoretic mobility shift analysis (EMSA) was performed using in vitro transcribed/translated PPARγ3 protein. An oligonucleotide containing a high-affinity PPRE, previously identified in the apo A-II promoter J site, was used in EMSA. This oligonucleotide was capable of binding the hPPARγ3/mRXRα heterodimers in EMSA (Fig. 4A, lanes 4–6). Homodimers of either hPPARγ3 or mRXRα, however, were incapable of binding to this oligonucleotide (Fig. 4A, lanes 2 and 3). These data hence prove that the protein transcribed from PPARγ3 mRNA is identical to the protein produced from the PPARγ1 mRNA in its capacity to bind to a PPRE.

Finally, we verified whether the human PPARγ3 cDNA was also capable of activating gene transcription through a PPRE. Therefore 3T3-L1 preadipocytes were cotransfected with the PPARγ3 expression vector pcDNA3-hPPARγ3 and a PPRE-driven luciferase reporter gene. The luciferase reporter gene was under the control of a multimerized ACO-PPRE site and the TK promoter (Fig. 4B). hPPARγ3 in the absence of ligand was incapable of activating this PPRE-based reporter. However, in the presence of ligand a 1.5-fold activation was ob-

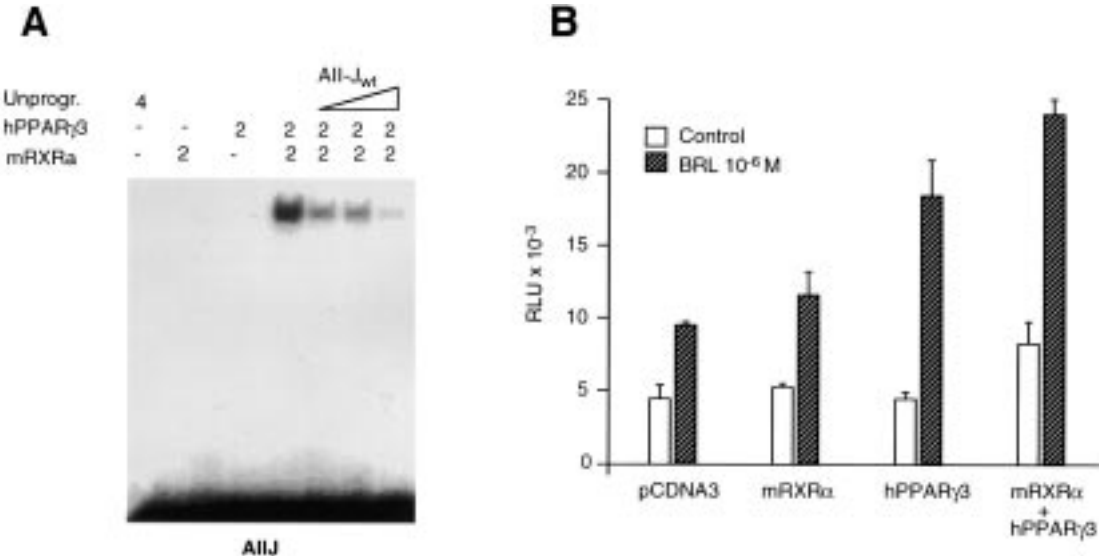

Fig. 4. Electrophoretic mobility shift analysis and transactivation assays demonstrating binding and transactivation of hPPARγ3/mRXRα heterodimers to a PPRE or PPRE driven reporter gene, respectively. A: hPPARγ3/mRXRα heterodimers bind to the AII-J_{wt} PPRE. EMSA was performed with end-labelled AII-J_{wt} PPRE oligonucleotide in the presence of in vitro transcribed/translated hPPARγ3 and mRXRα or unprogrammed reticulocyte lysate in the absence or presence of increasing concentrations of unlabelled wild-type AII-J_{wt} PPRE oligonucleotide as described in Section 2. B: Undifferentiated 3T3-L1 cells were cotransfected with the J_{wt3}-TK-CAT plasmid in the presence of an empty pSG5 expression vector or expression vectors encoding hPPARγ3, mRXRα, and both hPPARγ3 and mRXRα together. Cells were treated with BRL-49653 (1 μM), or vehicle (DMSO) and CAT activity was measured and expressed as described in Section 2.

served. This stimulatory effect was substantially enhanced when hPPARγ3 was cotransfected together with mRXRα. Upon the addition of the PPARγ ligand BRL-14653, luciferase expression was increased at least 2.5-fold when the cells were cotransfected with both hPPARγ3 and mRXRα. Similar results were obtained when prostaglandin J2 was used as a PPARγ ligand (data not shown).

4. Discussion

We have previously described the genomic structure of the human PPARγ gene and isolated the promoters of both PPARγ1 and PPARγ2 isoforms. While performing RNase protection assays, we have found an unexpected band whose length did not correspond with either the PPARγ1 or the PPARγ2 mRNA species, suggesting the existence of an alternative PPARγ mRNA subtype, containing only the untranslated exon A2, which we called PPARγ3. Several arguments support the hypothesis that the expression of PPARγ3 mRNA was directed by an independent promoter. First, primer extension assay indicates that there is a transcription initiation site for a novel PPARγ3 mRNA subtype, consistent with the observations of the RNase protection assay. Second, sequence analysis of the intronic sequence immediately upstream of this exon reveals the presence of different regulatory elements (TATA box, CCAAT box) which can direct gene expression, suggesting that these sequences could function as a promoter. Finally and most importantly, this region functions as a promoter in transient transfection experiments. Indeed, a fragment spanning 800 bp upstream of exon A2 was able to direct the expression of a heterologous luciferase reporter gene in transient transfection assays, demonstrating it functions as a promoter.

In agreement with previous studies, we show that the expression of the PPARγ gene is not restricted to adipose tissue. Whereas the PPARγ1 isoform has a relative ubiquitous expression, both PPARγ3 and PPARγ2 expression is relatively confined to certain tissues. In fact, in RNase protection assays PPARγ2 seems to be mostly restricted to adipose tissue whereas PPARγ3 is expressed in both adipose tissue and colon epithelium. In addition, in a previous manuscript we reported high level expression of PPARγ3 in human macrophages [17], which is consistent with the high level of activity of the PPARγ3 promoter in activated THP-1 cells. How the production of the different PPARγ subtypes is actually controlled is unclear at present but the presence of different promoters suggests that these different PPARγ subtypes might be important in tissue-specific regulation of gene expression. The observation that the tissues which reportedly express the highest amount of PPARγ, i.e. adipose tissue, macrophage, and colon, are also the only tissues which contain, in addition to the more ubiquitous PPARγ1 form, additional PPARγ types such as PPARγ2 and PPARγ3, suggests that these last might be responsible for more finely tuned control of gene expression. In addition, little is known about the function of the two different proteins transcribed from the PPARγ gene, i.e. PPARγ1 (translated either from the PPARγ1 and PPARγ3 mRNAs) and PPARγ2 (translated from the PPARγ2 mRNA). Although detailed side by side comparison of transactivation by PPARγ1 and PPARγ 2 has not been performed, conflicting evidence exists in the literature concerning differences in activity between PPARγ1 and -2. In fact, on the one hand it was shown that the NH-2 terminal of PPARγ2 might be a better ligand-independent activator relative to PPARγ1 [18]; on the other hand in other studies it was shown that both forms have a similar capacity to induce adipocyte differentiation [1,2].

In summary, in man there are three different subtypes of PPARγ mRNA transcribed from three different promoters. All three subtypes contain a common region spanning exons

1–6. In addition to the common exons, PPARγ1 contains exons A1 and A2, PPARγ2 exon B and PPARγ3 exon A2. PPARγ1 and PPARγ3 code for the same protein since exons A1 and A2 are untranslated, whereas PPARγ2 codes for a different protein which contains an additional 30 amino acids at its N-terminus encoded by exon B. Further studies are required to define the exact function of PPARγ in human physiology.

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