Original Article

A Promoter in the Novel Exon of hPPAR γ Directs the Circadian Expression of PPAR γ

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Aim: PPAR γ (peroxisome proliferator-activated receptor γ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors that regulate the expression of genes associated with lipid metabolism. Herein, we show that expression levels of the novel PPAR γ transcript exhibit circadian oscillation. To study the mechanisms controlling PPAR γ expression, a novel PPAR γ gene promoter was cloned and characterized.

Methods: We analyzed the novel PPAR γ promoter by luciferase reporter assays and gel shift analysis.

Results: Surprisingly, it was not an intron but rather the novel first exon of PPAR γ that was found to have functional minimal promoter activity. Luciferase reporter assays and gel shift assays revealed that the novel first exon is essential for novel PPAR γ promoter activation and that DBP (albumin gene D-site binding protein) and E4BP4 (E4 promoter A binding protein 4) bind directly to D-sites in the novel first exon.

Conclusion: Our results demonstrate that the PAR-bZIP (bZIP, basic leucine zipper) family and E4BP4 are the main regulatory factors involved in oscillation of novel PPAR γ expression. This regulatory mechanism clearly differs from that of the circadian expression of PPAR α .

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Introduction

In vertebrates, the PAR (proline- and acidic-amino-acid-rich) family of bZIP (basic leucine zipper) factors is composed of three proteins: hepatic leukaemia factor (HLF), D-site binding protein (DBP) and thyrotroph embryonic factor (TEF)¹⁻⁴. The PAR family has conserved basic regions flanked by PAR domains. Several other proteins have been identified

with characteristics similar to those of the PAR family. The mammalian transcription factor E4 binding protein 4 (E4BP4)⁵ has been identified as a member of the bZIP family of transcriptional factors; it contains a PAR factor-like basic domain including the extended basic region, but lacks a PAR region. Consistent with the similarity between their DNA-binding domains, these proteins have DNA-binding profiles very similar to those of PAR factors. Notably, although the PAR factors have been characterized as transcriptional activators⁶, E4BP4 has been reported to be a transcriptional repressor^{5,7}.

Organisms from bacteria to vertebrates display rhythms of activity that represent adaptations to daily changes in the environment. These rhythms are driven by endogenous oscillators called circadian clocks. Many

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of the key players in these oscillators are conserved between *Drosophila* and mice and, in both cases, the involvement of PAR and E4BP4 transcription factors has been described as well as the CLOCK/BMAL1 heterodimer. Since DBP is a transcriptional activator and E4BP4 is a repressor, their involvement may lead to more robust cycling or increased amplitude of target gene expression.

The PPARs (peroxisome proliferator-activated receptors) function as transcriptional regulators that govern metabolic homeostasis by serving as 'lipid sensors', responding both to dietary fatty acids and to their derivatives. PPAR γ is the third member of a subdivision within the broader nuclear receptor superfamily that includes PPAR α and PPAR β/δ ⁸⁻¹⁰. Differential promoter usage, coupled with alternate splicing of the PPAR γ gene, gives rise to a variety of mRNA isoforms (PPAR γ ^{1-4,9}). PPAR γ 1 exhibits widespread expression (e.g., in adipose tissue, pancreatic β cells, macrophages, vascular endothelium), albeit at low levels, while PPAR γ 2 is expressed almost exclusively in adipose tissue¹¹. PPAR γ 3 expression appears to be restricted to macrophages, adipose tissue and the colon^{12,13} and PPAR γ 4 remains to be defined¹⁴.

Oishi *et al.* have shown by *in vitro* studies that circadian expression of PPAR α is regulated directly by CLOCK protein via the E-box-rich region in the second intron¹⁵. Recently, PPAR γ was found to be rhythmically expressed in adipose tissue¹⁶. These reports suggest that circadian clock proteins play an important role in lipid metabolism by regulating the circadian transactivation of PPAR family members; however, which isoform(s) of PPAR γ can be expressed in oscillatory fashion, and the mechanism of the oscillatory expression of this gene, remain unclear. Herein, we measured the levels of novel PPAR γ mRNA in Caco2 cells after dexamethasone treatment and found circadian expression. To identify the circadian-related transcription factors that affect novel PPAR γ transcription, we analyzed the novel PPAR γ promoter by luciferase reporter assays and gel shift analysis. Surprisingly, we found that D-sites in the novel first exon of PPAR γ have functional promoter activity. Moreover, we found that the transcription factors DBP and E4BP4 bind directly to these D-sites and thereby regulate PPAR γ expression.

Materials and Methods

Cell Culture and Treatments

NIH 3T3 and HepG2 cells were maintained at 37°C in a humidified incubator with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) and 10%

fetal bovine serum (FBS). Caco2 were maintained in DMEM and 20% FBS, grown to 80% confluence and synchronized by the addition of 40 μ M dexamethasone. After 2h, the medium was changed to DMEM and 20% fetal bovine serum. Cells were maintained for another day prior to the start of experiments (at time 0), harvested every 4h, and total RNA was extracted.

For *in vitro* adipogenic induction, 3T3-L1 pre-adipocytes were grown to confluence in growth medium consisting of DMEM supplemented with 10% calf serum at 37°C. Differentiation was induced on day 0 (cells at 2 days postconfluence) by the addition of 10 μ g/mL insulin, 0.5 mM IBMX, and 0.25 μ M dexamethasone. After 48 h, the medium was replaced with DMEM containing 10% fetal bovine serum and 10 μ g/mL insulin only. Medium was changed every 48 h until cells had differentiated into mature adipocytes at 6 days.

Preparation of RNA Samples and Quantitative RT-PCR Analysis

RNA expression levels were determined using whole-cell or tissue RNA. RNA was extracted according to the reference¹⁷, and 0.5 μ g DNase-treated total RNA were reverse-transcribed using random hexamers and MuLV reverse transcriptase (Applied Biosystems). The cDNA equivalent to 20 ng total RNA was PCR-amplified in an ABI PRISM 7900 detection system (PE-Applied Biosystems¹⁸). The TaqMan probe numbers used are as follows: hBMAL1, Hs01546785_m1; hDBP, Hs00897003_m1; novel hPPAR γ , Hs01115729_m1; hGAPDH, Hs99999905_m1. Primers used are follow: hPPAR γ 1 Forward, 5'-ctcgaggacaccggagag-3', hPPAR γ 1 Reverse, 5'-aaaggctgactctcgtttgag-3', mPPAR γ 1 Forward, 5'-cggagggacgcggaagagag-3'; mPPAR γ 1 Reverse, tgtggcatccgcccacc, novel mPPAR γ Forward, 5'-gctgcagcgctaaattcttc-3', novel mPPAR γ Reverse, 5'-cgcacagtaaagggtagctcttg-3'; mGAPDH Forward, 5'-gcacagtcaaggccgagaat-3', mGAPDH Reverse, 5'-gccttctccatgggtgga-3'. The specificity of our assay was confirmed by sequencing PCR products.

RNAi Analysis

miR RNAi expression vector (Invitrogen) was delivered into Caco2 cells by a pulse of electroporation with a Nucleofector (Amaxa, Gaithersburg, MD). After electroporation, cells were immediately mixed with fresh medium for 10 min and then reseeded on plates for further experiments. The efficiency of the introduction of miR RNAi expression vector into cells was 80–90% as judged by the fluorescence intensity of

GFP. Empty vector (pcDNA6.2-GW/EmGFP-miR-nega) was used for negative control experiments. The nucleotide sequences of the engineered BLOCK-iTTM miR RNAi Select oligos targeting E4BP4 gene (Invitrogen) were TGCTGATGACTTTCCTACACAC-CATGTTTTGGCCACTGACTGACATGGTGT-GAGGAAAGTCAT (top) and CCTGATGACTTTC-CTCACACCATGTCAGTCAGTGGCCAAAACAT-GGTGTGGTAGGAAAGTCATC (bottom).

Construction of Novel PPAR γ Promoter Luciferase Plasmids

The transcriptional initiation site of novel PPAR γ (**Fig. 1A**) was reported (Gene Bank accession # NM_138711). The promoter fragment (−777 to +198) of the novel PPAR γ gene (**Fig. 1A**) was PCR-amplified from human genomic DNA (Gene Bank accession # NT_022517) and was cloned in front of the luciferase expression vector pGL3-Basic (Promega). This construct was designated p(−777, +198). A series of deletion constructs was produced using PCR or restriction sites in the promoter. Figure 1 shows the sequence of 5′-flanking −777 relative to the transcription initiation site, indicated as +1. Potential DBP recognition sites were identified using the program MatInspector (Genomatix GmbH, Germany).

Real-Time Luciferase Assay

Fragments of DNA containing the novel PPAR γ promoter region and its derivatives were cloned into pGL3-dLuc which contains a rapid degradation domain modified from mouse ornithine decarboxylase at the carboxy-terminal end of firefly luciferase¹⁹. After transfecting reporter plasmids using Lipofectamine 2000 (Invitrogen), NIH3T3 cells were stimulated with 100 nM dexamethasone for 2 h and then incubated with DMEM containing 0.1 mM luciferin (Promega), 25 mM HEPES (pH 7.2) and 10% FBS. Bioluminescence was measured and integrated for 1 min at intervals of 10 min using Kronos AB-2500 (ATTO).

Luciferase Assay

The internal control comprised reporter plasmids containing the novel PPAR γ promoter region (**Fig. 1**) or its derivatives co-transfected with TfxTM-50 (Promega) into NIH 3T3, HepG2 and Caco2 cells. Luciferase assays were performed as described previously²⁰. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and Luminometer Model TD-20/20 (Turner Designs). The transcriptional activities were normalized relative to Renilla luciferase activities.

Gel Shift Assay

Gel shifts were examined as described²¹. For construction of full-length DBP and E4BP4 expression plasmids, the total coding regions were ligated into pcDNA3. DBP and E4BP4 proteins were synthesized by coupled transcription-translation *in vitro* (TNT T7 Quick Coupled Transcription/Translation System, Promega). Nucleotide sequences of 30-bp oligonucleotide probes for D1, D2 and D3 sites were 5′-AGGGAGGAGCCTAAGGTAAGGAGTCAGAAA-3′, 5′-AGCTGCGGCTTTTATATAAGGTCA-GTGGTA-3′ and 5′-TAAGGTCAGTGGTAGGTA-AGGAAGGGGCCT-3′, respectively. These probes were end-labeled with [γ -32P]ATP using T4 polynucleotide kinase (New England BioLabs). The samples were resolved by electrophoresis on 4% polyacrylamide gels in 40 mM Tris-acetate, 1 mM EDTA and 5% glycerol at 150 V for 2 h.

Results

Circadian Expression of Novel PPAR γ mRNA in Caco2 Cells

Circadian expression of the PPAR α gene has been reported by Oishi *et al.*¹⁵. Their results strongly indicated that rhythmic expression of PPAR α mRNA is governed by binding of CLOCK/BMAL1 to an E-box in the second intron of PPAR α ; however, whether this mechanism governs the circadian expression of other members of the PPAR family, including PPAR γ and PPAR β/δ , remains unclear.

Recently, a new transcriptional initiation site of hPPAR γ has been identified (Gene Bank accession # NM_138711). The data showed that the first exon of novel hPPAR γ is located upstream from exon A2, which has been considered the first exon of hPPAR γ ^{3, 13} (**Fig. 1A**). The novel first exon was termed exon C (**Fig. 1A**, novel γ). Consequently, we used MatInspector (Genomatix) to search the genomic sequence for transcription factor binding sites both in exon C and in the genomic region upstream of exon C. We focused on transcription factors participating in the circadian regulation of gene expression and found three putative D-sites similar to the consensus D-site ([G/A]T[G/T]A[C/T]GTAA[C/T]) in exon C, but no E-box sequence (CACGTG) in exon C or the upstream genomic region (**Fig. 1B** and supplemental **Fig. S1**). Interestingly, exon C is conserved in mammals (98% sequence identity between humans and chimpanzees, 89% between humans and horses, 79% between humans and pigs, 77% between humans and mice/rats). Most notably, the sequence around the three putative D-sites is highly conserved (**Fig. 1B**);

Fig. 1. PPAR γ family.

(A) A scheme of the genomic structure of the 5'-flanking region of the human PPAR γ gene. Exon 1-6 are shared by all five subtypes. PPAR γ 1 contains in addition the untranslated exons A1 and A2. PPAR γ 2 contains exon B, which is translated, and PPAR γ 3 contains untranslated exon A2. PPAR γ 4 starts from exon E1. The novel PPAR γ contains untranslated exon C and A2. (B) Three putative DBP-binding sites on mammalian novel PPAR γ promoter regions. Sequence alignment of the region around putative DBP-binding sites indicate that D1- and D2-sites are well conserved between species. Conserved sequences are shown as white characters on black. The bases which are putative DBP-binding sequences are indicated by asterisks.

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-777 gatgagagct ggggagaagg gggctcggc tgaggggtcc ggggctgagg cagggtcag
-717 gtcgggcagg acccgactg acgggtcgcg ggcggggcgc tcacgggtga cgggtgaag
-657 gggctcttggg ctgagggcac ccgggctgag ggtcgctct accggagcgc gcactagggg
-597 cggagggcgc gggcacagag agccgcggcc ggtcgcgact cgagtggagc cgagccggtg
-537 cgtcccggtt tccaggacag ccccccctcg gagagcgccg ggtgggcgcg gagccgggga
-477 caecggggacc tccgcgtccc cggcggaggt gcgcaagcgt cgggagccgc tccgggggaa
-417 ctttgaggca ggggtgtctg acccggggaa gcgaagatcc gctgtcgctc gtcctgaggc
-357 cgcgcggcac cctgtctgag gaggaggtcc cgttcgctgt ccagagccca gggcgacccg
-297 ccgcttgggg acccggggac cgttcagcga gggcgcgctc tgtagctggc caggccctgt
-235 gccctgacgc gttgtctggt cctacgccc ggcggagggg ctggggacgg ggaactgggtg
-177 cctccgtgcc tccgcggatg gacgcgcag ccaggacccc cagccgcacc ccggggggct
-117 ggaaggcga agtcgctgct ttgtgggtc ctgcaagtgc caggcgctga ccactcctcc
-57 tgggagccta actgaggggg gttagggcag cagaggttaa cagaaatctc aggtcccttg
4 GTGGAAGGTG GGTGTGTAGT CGTGCTACTT TACGCTCGG TGTTAGGGA GGAGCCTAAG
64 GTAGGAGTC AGAAGCGGG AGTAACCGAG CTGCGCTTT TATATAAGG CAGTGTAAG
124 TAGGAAGGG GCTTAAOCT CTGCTGGTGA CCAGAAGCCT GCATTCTGC ATTCTGCTTA
184 ATTCCCTTC CTTAG

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Fig. S1. Nucleotide sequence of the 5'-flanking region and first exon (exon C) of the PPAR γ gene.

Nucleotide numbers are relative to the transcription initiation site. Putative binding sites for DBP are indicated in red.

however, less than 10% sequence conservation between humans and other species exists in the region upstream from exon C.

We initially investigated whether novel PPAR γ expression varies diurnally at the transcriptional level and examined the rhythmicity of novel PPAR γ gene expression. Oscillations in the expression of CLOCK genes in cultured Caco2 cells, which expresses PPAR γ abundantly (supplemental Fig. S2), were triggered by 2h treatment with dexamethasone. In Caco2 cells, novel PPAR γ transcripts were detectable at all times examined, and their abundance displayed a clear rhythm (Fig. 2A). We compared the circadian profile of novel PPAR γ mRNA with those of total PPAR γ and DBP mRNAs. The profiles of novel PPAR γ , total

Fig. S2. Expressions of PPAR γ in human cell lines (Caco2 and HepG2 cells).

Real-time PCR was used to detect the expressions of total hPPAR γ , hPPAR γ 1 and a novel hPPAR γ . Notably, the novel hPPAR γ mRNA expression in Caco2 cells was apparently high. The relative levels of each mRNA were normalized to the corresponding hGAPDH levels. Each value is the mean $\pm$ SD of three replicates for a single assay.

Fig. 2. Transcriptional oscillation of novel PPAR γ .

(A) Temporal expression pattern of novel PPAR γ , total PPAR γ and DBP genes in Caco2 cells after dexamethasone treatment were studied. Total RNAs were isolated at each time point. Quantitative real-time RT-PCR was performed using novel hPPAR γ , total PPAR γ , hDBP, and hGAPDH specific primers. The relative levels of each mRNA were normalized to the corresponding hGAPDH levels. Each value is the mean $\pm$ SD of three replicates for a single assay. The peak values are adjusted to 1.0. Typical profiles are shown in the figure with similar results in two separate determinations. (B) DBP is a positive regulator and E4BP4 is a negative regulator of novel PPAR γ transcription. Caco2 cells were transfected with DBP-expression vector or pcDNA3.1 (as a negative control), and E4BP4-miR RNAi expression vector or pcDNA6.2-GW/miR (as a negative control). At 24 h after transfection, DBP, E4BP4 and novel PPAR γ mRNA levels were determined by quantitative PCR. (C) Transcriptional oscillation of novel PPAR γ was monitored using a cell culture-based luminescence reporter assay. Promoter fragments of the novel PPAR γ gene ranging from -777 to +198 (p(-777, +198)) were inserted into the pGL3-Basic luciferase vector. NIH3T3 cells were transfected with p(-777, +198) and stimulated with dexamethasone. Bioluminescence was measured and integrated for 1 min at intervals of 15 min. Ordinate and abscissa represent relative counts and time after dexamethasone, respectively. 1440 minutes = 1 day.

PPAR γ and DBP mRNAs were nearly identical (**Fig. 2A**). In contrast, the profiles of novel PPAR γ mRNA and CLOCK mRNA were essentially out of phase (data not shown). These data suggest that the circadian expression of novel PPAR γ in Caco2 cells is directly associated with DBP expression and indirectly associated with CLOCK expression. To determine whether DBP regulates novel PPAR γ transcription, we transfected Caco2 cells with a DBP expression vector (pcDNA3.1 DBP). Overexpression of DBP resulted in increases in DBP mRNA and novel PPAR γ mRNA of approximately 8-fold and 3-fold, respectively, as compared with controls (**Fig. 2B**), suggesting that DBP positively regulates novel PPAR γ transcription in Caco2 cells. Next, we evaluated the effect of E4BP4

on activation of the novel PPAR γ transcription, since E4BP4 has been reported to also bind to the D-site²²⁾ and to be a transcriptional repressor^{5, 7)}.

Knock-down of E4BP4 resulted in a decrease in E4BP4 mRNA and an increase in novel PPAR γ mRNA of approximately 0.5-fold and 4-fold, respectively, as compared with negative controls (**Fig. 2B**), suggesting that E4BP4 negatively regulates novel PPAR γ transcription in Caco2 cells.

Analysis of the 5'-Flanking Region of the Human Novel PPAR γ gene

To examine the transcriptional oscillation of novel PPAR γ , 1.0 kb of the novel hPPAR γ promoter sequence containing exon C was cloned (supplemental

Fig. S1), and a real-time luciferase reporter assay was performed.

NIH3T3 cells were transfected with the 5'-flanking region of novel hPPAR γ (from -777 to +198) fused to luciferase (supplemental Fig. S1) and then stimulated with dexamethasone as described in Materials and Methods. After this stimulation, light emission in the presence of luciferin was measured and integrated over 1 min at intervals of 15 min, revealing a circadian rhythm of luciferase activity (Fig. 2C). This result indicates that the promoter region used in the real-time luciferase reporter assay is sufficient to produce circadian transcriptional oscillation.

Different Effects of PAR Proteins, E4BP4 and CLOCK/BMAL1 on Novel PPAR γ Promoter Activity

To determine whether the putative D-site elements are functional, we conducted luciferase assays with a construct of the novel PPAR γ promoter fused to the reporter gene vector pGL3-Basic, which we termed p(-777 to +198). The p(-777 to +198) plasmid transfected into NIH 3T3 cells with the DBP expression vector stimulated luciferase activity in a dose-dependent manner (Fig. 3). Interestingly, despite the absence of an E-box in this region, the CLOCK/BMAL1 expression vector significantly activated the novel PPAR γ promoter, as did the DBP expression vector, although the effect of CLOCK/BMAL1 was weaker than that of DBP (Fig. 3). These reporter assays suggest that CLOCK and BMAL1 are indirectly involved in activation of the novel PPAR γ promoter. Next, we evaluated the effect of E4BP4 on activation of the novel PPAR γ promoter, since E4BP4 has also been reported to bind to the D-site²². The transcription activities of the novel PPAR γ promoter were dose-dependently suppressed by transfection of increasing amounts of the E4BP4 expression vector. Together, the data suggest that these putative D-sites are involved in positive or negative regulation of novel PPAR γ expression.

Regulation of the Novel PPAR γ Gene by the First 5' Untranslated Exon

Next, to functionally characterize novel PPAR γ promoter (Fig. 1A) activity, a series of promoter deletions was constructed in pGL3-Basic. Transfection of the p(-777 to +198) plasmid into NIH 3T3 cells with or without the DBP expression vector stimulated luciferase activity approximately 9-fold and 14-fold, respectively, as compared with pGL3-Basic (no promoter insert) (Fig. 4A). Interestingly, the construct containing the sequence from -559 to +198 showed the highest endogenous and DBP-induced stimulation

Fig. 3. Transcriptional regulation of the novel PPAR γ promoter by DBP, CLOCK/BMAL1, HLF, and E4BP4 protein alone.

A reporter plasmid (10 ng) containing PPAR γ gene ranging from -777 to +198 (p(-777, +198)), and increasing doses of each expression plasmid, were cotransfected into NIH3T3 cells. Transfection efficiency was normalized by cotransfection of pRL-TK. Promoter activity is shown as a factor in the induction of luciferase over background activity measured in cells transfected with pGL3-Basic alone. Each value is the mean $\pm$ SD of three replicates for a single assay. The results shown are representative of three independent experiments.

of activity, approximately 14-fold and 19-fold, respectively, as compared with pGL3-Basic, suggesting that the promoter has active silencer elements at -777 to

Fig. S3. Expressions of PPAR γ s in mouse tissues and cell lines.

Real-time PCR was used to detect the expressions of total mPPAR γ , mPPAR γ 1 and novel mPPAR γ . The relative levels of each mRNA were normalized to the corresponding hGAPDH levels. Each value is the mean $\pm$ SD of three replicates for a single assay.

-559. Deletion of the sequence between nucleotides -559 and -22 mildly decreased luciferase activity. In contrast, deletion from -22 to +28 had no adverse effect on luciferase activity. These data suggest that at least one DBP binding site governing novel PPAR γ expression lies between +28 and +198, as speculated, and that there are silencer and enhancer elements between -777 and -22. Another possibility is that more than one DBP binding site is located somewhere in the -777 to -22 sequence.

Next, we constructed a series of oligonucleotides from which the sequence between +30 and +198, containing the three putative D-sites, had been deleted, and transiently transfected NIH3T3, HepG2 and Caco2 cells (**Fig. 4B** and supplemental **Fig. S4**). Surprisingly, removal of the sequence from +30 to +198 totally abolished endogenous as well as DBP-induced promoter activity in all cell lines tested. These data indicate that the first exon containing all putative

Fig. 4. Transcriptional activities of the novel PPAR γ promoter deletions cotransfected with or without DBP.

(A) Analysis of novel PPAR γ promoter function in NIH3T3. NIH3T3 cells were transiently transfected with the chimeric promoter constructs ranging from -777 to +28 relative to the transcription start site and extending to +198 plus either DBP expression plasmid (closed columns) or pcDNA3.0 as a control (open columns). Three asterisks indicate the three putative D-sites. (B) NIH3T3 cells were transiently transfected with the chimeric promoter constructs ranging from -777 to -22 relative to the transcription start site and extending to +29 plus either DBP expression plasmid (closed columns) or pcDNA3.0 as a control (open columns). Three asterisks indicate the three putative D-sites. Each value is the mean $\pm$ SD of three replicates for a single assay. The results shown are representative of three independent experiments.

D-sites is a minimal promoter region, whereas the genomic region upstream of the first exon is not essential for the promoter activity of novel PPAR γ . The expression level of novel PPAR γ in HepG2 cells is quite low, whereas the expression level of novel PPAR γ in Caco2 cells is high (data not shown). Therefore, the effect of DBP on novel PPAR γ promoter constructs is independent of the endogenous expression levels of PPAR γ in these cell lines (**Fig. 4B** and supplemental **Fig. S1**).

All Three D-Sites on the First Exon of Novel PPAR γ are Involved in Transcriptional Regulation

To test whether the putative D-sites actually interact with DBP and E4BP4 for transcriptional regulation, we compared the transcriptional activity of

Fig. S4. Transcriptional activities of the novel PPAR γ promoter deletions cotransfected with or without DBP in HepG2 or Caco2.

HepG2 cells or Caco2 cells were transiently transfected with chimeric novel PPAR γ promoter constructs ranging from -777 to +198 or from -777 to +29. DBP expression plasmid (closed columns) or pcDNA3.0 as a control (open columns). Promoter activity is shown as a factor in the induction of luciferase over background activity measured in cells transfected with pGL3-Basic alone. The C exon is indicated in bold. Three asterisks indicates the three putative D-sites. Each value is the mean $\pm$ SD of three replicates for a single assay.

p(-22, +198) and a construct of p(-22, +198) from which putative D-sites had been deleted, which we termed p(-22, +198)null. The p(-777, +198), p(-22, +198) and p(-22, +198)null constructs were cotransfected with the DBP and E4BP4 expression plasmids (**Fig. 5A**). The DBP expression plasmid increased the reporter activity of p(-22, +198) as well as that of p(-777, +198). The E4BP4 expression plasmid not only suppressed the basal activities of these two promoters, but also significantly reduced DBP-mediated transcription (**Fig. 5A**). In contrast, the reporter activity of the p(-22, +198)null construct remained at the basal level and did not change significantly in any of the experiments. These results indicate that the putative D-sites actually interact with DBP and E4BP4 during transcriptional regulation.

To determine which D-sites are involved in transcriptional activity, we generated a series of mutant constructs of p(-22, +198) and used them in luciferase assays (**Fig. 5B**). Compared with p(-22, +198)null, the rates of transcriptional activation by DBP and E4BP4 were significantly higher in p(-22, +198)D1, p(-22, +198)D2 and p(-22, +198)D3 (**Fig. 5B**). These results suggest that all three D-sites function in DBP- or E4BP4-mediated transcriptional expression of novel PPAR γ *in vitro*.

DBP and E4BP4 Bind Mainly to the D2 Site

To clarify the importance of the D-sites for transcriptional regulation of the novel PPAR γ promoter (**Fig. 1A**) through the DNA-binding activities of DBP

and E4BP4, we performed gel shift assays using DBP and E4BP4, synthesized by coupled transcription-translation *in vivo*, and probes containing the putative D-sites. We used end-labeled DNA fragments of 30 bp containing D1, D2 and D3 site probes (**Fig. 6A, B**). As a positive control, consensus binding sequences for both DBP and E4BP4 (5'-GCCCCGCTACATATACGTAACAAGCGTTCGC-3')²²⁾ were used. We observed shifted bands for all three probes, in particular the D2 site, in the presence of DBP or E4BP4, and all of the bands disappeared in the presence of unlabeled competitors. These results indicate that the formation of protein-DNA complexes is specific for the DBP- and E4BP4-binding sites and that the D2 site has higher affinity than the D1 or D3 sites for the two proteins (**Fig. 6B**). In contrast, promoter reporter assays show that not only the D2 site, but also the D1 and D3 sites, function to mediate the effects of DBP and E4BP4 on the transcriptional regulation of novel PPAR γ . A possible explanation is that the first exon may contain additional DBP- and E4BP4-specific regulatory elements that cooperate with the D-sites to regulate transcription of the novel PPAR γ gene (**Fig. 5B**).

We conclude that novel PPAR γ transcription is directly regulated by binding DBP and E4BP4 to the three D-sites in the first exon, although the highest binding affinity to the D-site does not correspond exactly to the highest transcriptional activation of the novel PPAR γ promoter *in vitro*.

Discussion

First exons play important roles in basal transcription of some genes^{23, 24)}, and we now show that the first novel exon of PPAR γ fits this pattern (**Fig. 1**). Deletion of the first exon from the reporter constructs totally abolished basal promoter activity (**Fig. 4B**), whereas a construct containing only the first novel exon C showed significant transcriptional activity in NIH 3T3, HepG2 and Caco2 cells (**Fig. 4B** and **Fig. S2**). Therefore, we conclude that the first exon C of the PPAR γ gene is essential for promoter activity, whereas the genomic region upstream of the first exon contributes to modulation of promoter activity but is not obligatory. This regulatory mechanism of the novel PPAR γ gene may be evolutionarily conserved, since high levels (>75%) of sequence conservation in the first exon C are observed among different species from humans to mice, and low levels (<10%) of conservation are observed in the sequence upstream from the first exon C (**Fig. B**).

To date, there have been no reports describing

Fig. 5. Analysis of DBP and E4BP4 binding to D1-, D2- and D3-sites of novel PPAR γ promoter.

(A) Transcriptional regulation of the novel PPAR γ promoter by the coexpression of DBP and E4BP4. NIH 3T3 cells were transfected with constructs derived from novel PPAR γ gene ranging from -777 to +198 (p(-777, +198)) or -22 to +198 (p(-22, +198)). A plasmid that three putative DBP binding sites were deleted from the p(-22, +198) is also constructed (p(-22, +198) null) and used. Presence (+) or absence (-) of DBP-, and E4BP4-expression plasmids (125 and 400 ng, respectively) is noted. The total amount (400 ng) of expression plasmids per well was adjusted by adding a pcDNA3.0 plasmid. Promoter activity is shown as the factor of induction of luciferase over background activity measured in cells transfected with pGL3-Basic alone. (B) NIH3T3 cells were transfected with ranging from -22 to +198 (p(-22, +198)), and its D-sites mutants. Presence (+) or absence (-) of DBP- and E4BP4-expression plasmids (250 and 400 ng, respectively) is noted. Promoter activity is shown as a factor in the induction of luciferase over background activity measured in cells transfected with pGL3-Basic alone. Each value is the mean $\pm$ SD of three replicates for a single assay. The results shown are representative of three independent experiments. We used Student's *t*-test to examine the significance of differences (* p < 0.05, ** p < 0.01, *** p < 0.001).

regulation of the promoter region for the novel human PPAR γ gene. Our analysis indicates a relationship between novel PPAR γ and the circadian clock. The transcriptional activators CLOCK and BMAL1 drive expression of the DBP gene²⁵⁾, and we found that DBP promotes, whereas E4BP4 inhibits, novel PPAR γ gene activation (**Fig. 7**). This regulation may increase

the amplitude of the oscillation of novel PPAR γ gene expression. On the other hand, expression of the PPAR α gene is driven directly by CLOCK/BMAL1 complexes through interaction with the E-box-rich region¹⁵⁾. These distinct mechanisms of regulation might be one of the reasons why the various PPARs show distinct rhythms of expression in the same tissue

Fig. 6. Gel shift assay showing that putative D-sites bind to DBP and E4BP4.

(A) DBP or E4BP4 protein was incubated with radiolabeled oligonucleotides and analyzed by electrophoretic mobility shift assay. Bands shown are the gel shift complexes formed with DBP or E4BP4. Lane 1 and lane 4, no competitor (-); lane 2, 100-fold excess of wild type competitor DNA (WT); lane 3, 100-fold excess of mutant competitor DNA (MT); lane 4, no protein. D1, D2 and D3 shown represent radiolabeled oligonucleotides containing the three putative D-sites. (B) C, D1, D2 and D3 represent radiolabeled oligonucleotides containing the consensus D-site and the three putative D-sites, respectively. Radioactive bands corresponding to bound and free forms were shown by a solid and broken arrow, respectively. The D2 site has higher affinity than the D1 or D3 sites for the two proteins.

*in vivo*¹⁶.

The gel shift assays showed that DBP and E4BP4 proteins bind more strongly to the D2 site than to the D1 or D3 site. The different nucleotide sequences of the sites may account for the different DNA-binding specificities of DBP or E4BP4 protein. In contrast, the promoter reporter assays showed that not only the D2 site but also the D1 and D3 sites function to mediate the effects of DBP and E4BP4 on the transcriptional regulation of novel PPAR γ . A possible

Fig. 7. Links between circadian clock proteins and ppar genes expression.

The transcriptional activators CLOCK and BMAL1 drive the expression of the DBP gene, and then DBP activates transcription of the novel PPAR γ gene, whereas E4BP4 suppresses this. On the other hand, the expression of the PPAR α gene is driven by the CLOCK/BMAL1 complexes directly.

explanation is that the first exon contains additional DBP- and E4BP4-specific regulatory elements that cooperate with the D-sites to regulate transcription of the novel PPAR γ gene.

Differential splicing of novel PPAR γ gives rise to four distinct mRNA isoforms, called PPAR γ 1, 2, 3, 4 and novel (Fig. 1). The PPAR γ 1, 3, 4 and novel mRNA species code for the same 477 amino-acid protein. Although the expressed protein is the same for each mRNA isoform, the differences in the promoters and noncoding exons are thought to result in differential tissue expression. Deletion of the three D-sites from the reporter construct containing the novel PPAR γ promoter decreases its activity nearly to that of the basal control plasmid (pGL3 basic) (Fig. 5A, 5B), and the effect of DBP or E4BP4 on the reporter construct is independent of the endogenous expression level of novel PPAR γ in all cell lines tested (supplemental Fig. S2, S4). These results indicate that tissue-specific expression of novel PPAR γ , as well as circadian expression, is controlled mainly through interaction of the D-sites of the first exon with proteins such as PAR or E4BP4.

The specific role of novel PPAR γ *in vivo* remains unclear. The novel PPAR γ is expressed abundantly in adipose tissue (data not shown) and PPAR γ is known to be the key regulator of adipocyte differentiation⁹. Therefore, knowledge of the factors that govern diurnal expression of the novel PPAR γ gene might be of crucial importance to understanding the link between the circadian clock and adipocyte differentiation.

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