

Intranuclear Localization of EGFP-mouse PPAR γ 1 in Bovine Fibroblast Cells

Sorayya Ghasemi, B.Sc.¹, Kamran Ghaedi, Ph.D.^{1,2*}, Mohammad Hossein Nasr Esfahani, Ph.D.^{2*}, Somayye Tanhaei, M.Sc.², Farzaneh Rabeii, B.Sc.², Khadijeh Karbalaii, M.Sc.², Hossein Baharvand, Ph.D.³, Abolghasem Esmaeili, Ph.D.¹

1. Biology Department, School of Sciences, University of Isfahan, Isfahan, Iran
2. Department of Cell and Molecular Biology, Royan Institute for Animal Biotechnology, ACECR, Isfahan, Iran
3. Department of Stem Cells and Developmental Biology, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

* Corresponding Address: P.O.Box: 19395-4644, Department of Cell and Molecular Biology, Royan Institute for Animal Biotechnology, ACECR, Isfahan, Iran

Emails: kamranghaedi@royaninstitute.org, mh.nasr-esfahani@royaninstitute.org

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Abstract

Objective: The aim of this study was to clone PPAR γ 1 cDNA in an appropriate mammalian expression vector, with a chimeric cDNA form, encompassing PPAR γ with enhanced green fluorescent protein (EGFP) cDNA. This recombinant plasmid will be used for further analyses to investigate the molecular mechanism of PPAR γ 1 for neural differentiation process. Moreover, the nuclear localization of the PPAR γ 1 protein linked to EGFP marker was chased by using transient transfection of a constructed plasmid into bovine fibroblast cells.

Materials and Methods: Total RNA was extracted from the fatty tissue of an adult mouse. Using specific pair primers, PPAR γ 1 cDNA was synthesized and amplified to produce the entire length of ORF. RT-PCR products containing PPAR γ 1 cDNA were treated by enzymatic digestion and inserted into the pEGFP-C1 downstream from EGFP cDNA. The constructed vector was used for transformation into bacterial competent cells. Positive colonies which showed inserted PPAR γ 1 cDNA were selected for plasmid preparations and additional analysis was performed to ensure that PPAR γ 1 cDNA was inserted properly. Finally, to confirm the intracellular localization of EGFP-PPAR γ 1, bovine fibroblast cells were transfected with the recombinant plasmid.

Results: Our results from enzymatic digestion and sequencing confirmed, as expected, that PPAR γ 1 cDNA was amplified and cloned correctly. This cDNA gene encompassed 1428 bp. The related product was entered into the nucleus of bovine fibroblasts after transfection of its cDNA.

Conclusion: PPAR γ 1 cDNA was cloned and sorted into nuclear compartments of bovine fibroblast cells upon transfection.

Keywords: PPAR γ , Nuclear Targeting, Enhanced Green Fluorescent protein, Cloning, Transfection

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Introduction

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors that mainly act as transcription factors which control regulation of the expression of specific genes (1). Thus PPARs exert their regulations on various cellular functions including cellular differentiation, development, and metabolism in mammals (2, 3). Three types of PPARs have been identified so far: alpha, beta/delta and gamma. PPAR α (alpha) is expressed mainly in liver, kidney, heart, muscle, and adipose tissue; while β/δ (beta/delta) show expression in a broad range of tissues, markedly in the brain. In adipose tissue, PPAR γ (gamma) expression is high. PPARs have been originally identified in *Xenopus* as a type of

receptor which induces the proliferation of peroxisomes in cells (4). The best-known PPAR ligands are thiazolidinediones (5). All PPARs heterodimerize with the retinoid X receptor (RXR) and bind to specific regions on the DNA of target genes. DNA sequences of target genes are termed PPREs (peroxisome proliferator hormone response elements) which occur in the promoter of targeted genes with a consensus sequence like "AGGTCAXAG-GTCA", (X is any nucleotide). Thus PPARs cause an increase or decrease in the transcription rates of target genes, depending on the gene's function (6). The net functions of PPARs are modified by their ligand-binding domains which interact by a number of coactivator and corepressor proteins (7).

Free fatty acids and eicosanoids are among endogenous ligands for PPARs. The molecular structures of PPARs are comprised of the following domains: (A/B) N-terminal region, (C) DNA-binding domain (DBD), (D) flexible hinge region, (E) ligand binding domain (LBD) and (F) C-terminal region. DBD contains two zinc finger motifs which bind to specific sequences of DNA known as hormone response elements when the receptor is activated. The ligand binding domain contains an extensive secondary structure consisting of 13 alpha helices and a beta sheet (8, 9). Natural and synthetic ligands bind to LBD, either activating or repressing the receptor (10, 11).

As noted earlier, one of the members of the PPAR family is PPAR γ . In mammals two PPAR γ isoforms, PPAR γ 1 and PPAR γ 2, have been detected (12). Both isoforms are abundantly expressed in adipose tissue. PPAR γ 1 is detected at a lower level of expression in liver and heart tissues, while in skeletal muscle both types are expressed at low levels (13). PPAR γ plays a key role in adipogenesis and adipocyte gene expression, and it is the receptor for the thiazolidinedione class of insulin-sensitizing drugs (12). PPAR γ exerts its function by binding to PPRE at the promoters of target genes. The mouse PPAR γ gene has nine exons and extends more than 100 kilobases. Alternate transcription start sites and alternate splicing generate PPAR γ 1 and PPAR γ 2 mRNAs, which differ at their 5'-ends. Thus PPAR γ 1 is encoded by eight exons, whereas PPAR γ 2 is encoded by seven exons. The 5'-untranslated sequence of PPAR γ 1 is comprised of exons A1 and A2, whereas that of PPAR γ 2 plus the additional PPAR γ 2-specific N-terminal amino acids are encoded by exon B, located between exons A2 and A1. The remaining six exons, termed 1 to 6, are common to both PPAR γ 1 and γ 2 (13). Due to various functions which are suggested for PPAR γ , cloning of related cDNAs seems to be necessary since there is no evidence for the role of exogenous PPAR γ in the process of neural cell differentiation. Thus, the aim of this study is to clone PPAR γ 1 cDNA in a mammalian expression vector in a chimeric cDNA type, encompassing PPAR γ with enhanced green fluorescent protein (EGFP) cDNA and determine the nuclear localization of the PPAR γ protein when fused to an EGFP marker.

Materials and Methods

RNA extraction

RNA was purified from the fatty tissue of a mouse (Souris strain) using RNX-PLUS kit (Cinnagen, Iran) as follows: 1ml RNX solution was added to 5mg of wet tissue and the tissue was homogenized.

At the next step, 200 μ l chloroform was added to the homogenized tissue sample. After vigorous shaking and sedimentation at 13000 rpm for 15 minutes at 4°C, the upper phase was transferred to a fresh tube. RNA was precipitated by adding the same volume of isopropanol and centrifugation again at 13000 rpm for 15 minutes at 4°C. The RNA solution was washed with 75% ethanol and dissolved in DEPC treated water. Total RNA concentration and quality was evaluated with OD absorption at 260 nm with a CE7250 spectrophotometer (Bioaquarius, UK) and agarose-gel electrophoresis.

cDNA synthesis and RT-PCR condition

In order to remove DNA contamination, 2 μ g of extracted RNA was treated with DNaseI (Fermentas, Lithuania) for 30 minutes at 37°C. Then cDNA was synthesized using a cDNA synthesis kit (Fermentas, Lithuania) according to the manufacturer's protocol. Random hexamer primers (Fermentas, Lithuania) were used in this study. RT-PCR for PPAR γ 1 cDNA amplification was done with 2 μ l of first stranded cDNA in an Eppendorf Mastercycler gradient thermal cycler (Eppendorf, Germany) using EX-taq DNA polymerase (Takara, Japan) as described below:

SOE-RT PCR (splicing by overlap extension-RT PCR) was used for amplifying the entire length of PPAR γ 1 cDNA. The SOE-RT PCR was performed in two-step PCR reactions as described below:

Step1: The first PCR step PCR was set to amplify two different fragments of PPAR γ 1 cDNA covering the whole length of related cDNA. The length of first fragment was 1033 bp which was amplified with primer pairs PPAR γ 1-F SacI and PPAR γ 1-R-1021 (introducing SacI restriction site at the 5' end). A second 886 bp fragment was obtained in a PCR reaction with PPAR γ 1-R-KpnI and PPAR γ 1-F-552 primer pair (introducing KpnI restriction site at the 3' end). Both fragments were purified by the QIAprep Spin Miniprep kit (Qiagen, Germany) and used as the template for the next step.

Step2: The final stage for amplifying PPAR γ 1 cDNA was a set of PCR with the last step products as template and using primer pairs PPAR γ 1-F-SacI and PPAR γ 1-R-KpnI to produce the full length PPAR γ 1 cDNA (1428 bp). Moreover, in a different RT-PCR reaction with B-tubulin F and B-tubulin R primers, and with using a 2 μ l cDNA template; we amplified a 318 bp fragment of B-tubulin cDNA as a housekeeping gene to control RT-PCR cDNA synthesis steps.

Plasmid constructions

In order to provide a suitable amount of PPAR γ 1 cDNA, amplified PCR products were inserted into a pTZ57R/T vector (Fermentas, Lithuania, catalog # K1214) and transformed into *E. coli* competent cells. After blue/white colony selection, several positive colonies were chosen for plasmid extraction and sequence PPAR γ 1 cDNA analyses (Bioneer, Korea). Sequence checked recombinant plasmid was digested with *SacI* (Fermentas, Lithuania) and *KpnI* (Fermentas, Lithuania). The *SacI-KpnI* fragment which contained PPAR γ 1 cDNA was ligated with a pEGFP-C1 vector at the related sites. Ligation was carried out according to the Takara Ligation kit, (TaKaRa, Japan). Ligation mixture was transformed into competent *E. coli* TOP10 (Invitrogen, USA). A colony-PCR experiment was done to isolate the recombinant pEGFP vector which contained a chimeric cDNA of PPAR γ 1 and EGFP termed pEGFP/ EGFP- PPAR γ 1. Whole steps are represented (Fig 1).

Cell culture and transient transfection conditions

Bovine fibroblast cells were cultured in 10% DMEM-FCS (Gibco, USA) supplemented with 100 U/ml penicillin under a humidified atmosphere at 5% CO₂.

Bovine fibroblast cells (15000 cells/well) were plated in 24-well plates. Cells were grown on sterile glass coverslips in 24-well plates (TPP Company, Switzerland) and transfected with 800 ng of plasmid using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. The

50 μ l DNA-Lipofectamine complex was added to 250 μ l Opti-MEM I medium (Gibco, USA) pre-washed cells and incubated for 6 hours, at 37°C.

Fluorescence Microscopy

Two days post transfection, cells were washed in PBS and fixed for 30 minutes with 4% paraformaldehyde (Sigma, USA) in PBS. The cells were mounted with entellan (Merck, Germany). Fluorescence images were obtained using a U-LH100-HGAPO Olympus (BX51, Japan) fluorescence microscope.

Results

Target gene amplification

Total RNA, from mouse fatty tissue which has been reported to show high expression of PPAR γ , was extracted. The integrity of extracted RNA was evaluated after agarose gel electrophoresis. Three distinct ribosomal RNAs appeared in the gel as sharp bands (data not shown). To ensure the presence of cDNA synthesis stage, RT-PCR of a housekeeping gene, B-tubulin5, was done by using related specific primers (Table 1) that resulted in a sharp 318bp band which was absent in the control sample (Fig 1A, lane 1).

RT-PCR was successful for amplifying two fragments of PPAR γ 1 cDNA. Product sizes were as expected (Fig 2B, lanes 1 and 2). As previously described in materials and methods, the entire length of PPAR γ 1 cDNA (1428 bp) was produced at the second step of SOE-PCR (Fig 2C) and cloned into a pTZ57R/T vector termed pTZ57R/T/ PPAR γ 1 cDNA.

Table1: List of primers

PRIMER NAME		PRIMER SEQUENCE	Product length	Annealing temperatures used for PCR
Beta tubulin	F	5'- TCACTGTGCTGAACTTACC -3'	318 bp	63°C
Beta tubulin	R	5'- GGAACATAGCCGTAAACTGC -3'		
PPAR γ 1-SacI	F	5'- ATTTGAGCTCAAGTTGACACAGAGATGCCATTCTG-3' <i>SacI</i>	1033 bp	65°C
PPAR γ 1-1021	R	5'-GATGGAGTCCTCATCTCAGAGG-3'		
PPAR γ 1-552	F	5'-GCCAACAGCTTCTCCTTCTCGGCC-3'	786 bp	60°C
PPAR γ 1-KpnI	R	5'- AATTGGTACCCTAATACAAGTCCTTGATAGATC -3' <i>KpnI</i>		
PPAR γ 1-SacI	F	5'- ATTTGAGCTCAAGTTGACACAGAGATGCCATTCTG-3' <i>SacI</i>	1450 bp	70°C
PPAR γ 1-KpnI	R	5'- AATTGGTACCCTAATACAAGTCCTTGATAGATC -3' <i>KpnI</i>		
EGFP-C1	F	5'- AACGAGAAGCGGATCACATGC -3'	676 bp	63°C
PPAR γ 1-575*	R	5'- GGCCGAGAAGGAGAAGCTGTTGGC -3'		

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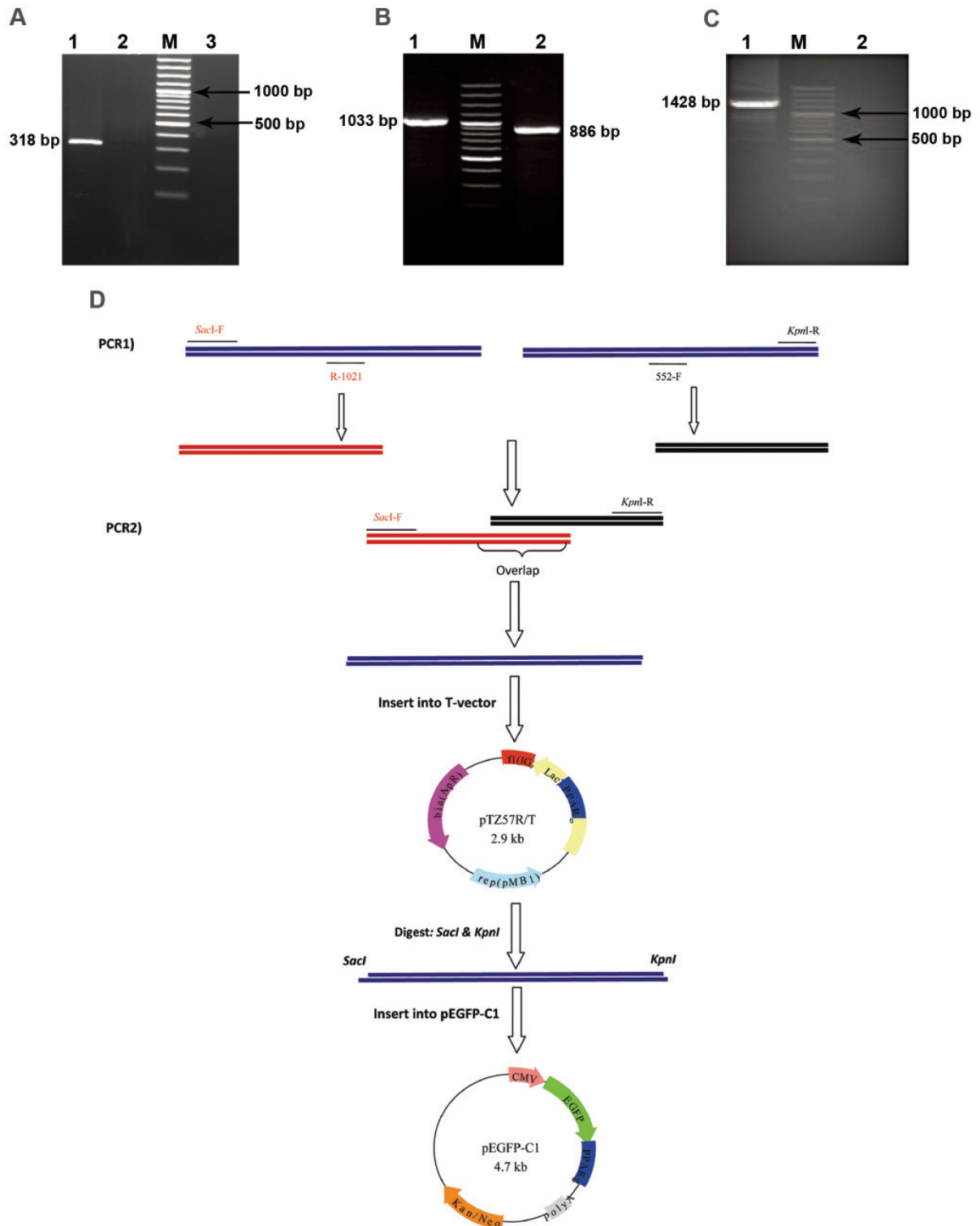


Fig 1: RT-PCR products of *b-tubulin* and *PPAR γ 1* fragments. **A.** Partial fragment of amplified *b-tubulin* cDNA (lane 1), lane 2 is the control sample, lane 3 is blank; M is the marker (100 bp: Fermentas, Lithuania). **B.** Two amplified fragments of *PPAR γ 1* cDNA (lanes 1, 2); M is the marker (100 bp: Fermentas, Lithuania). **C.** The entire amplified *PPAR γ 1* cDNA (lane 1); lane 2 is the negative control; M is the marker (100 bp: Fermentas, Lithuania). **D.** Schematic representation of the strategy for cloning *PPAR γ 1* cDNA.



Fig 2: Schematic representation of PPAR γ 1 cDNA and protein sequences. A. ORF sequence of PPAR γ 1 cDNA. B. Produced amino acid residues of PPAR γ 1 cDNA. C. Partial sequence of PPAR γ 1 cDNA representing the 5'-part of PPAR γ 1 cDNA and SacI restriction site. D. Partial sequence of PPAR γ 1 cDNA representing the 3'-part of PPAR γ 1 cDNA and KpnI restriction site.

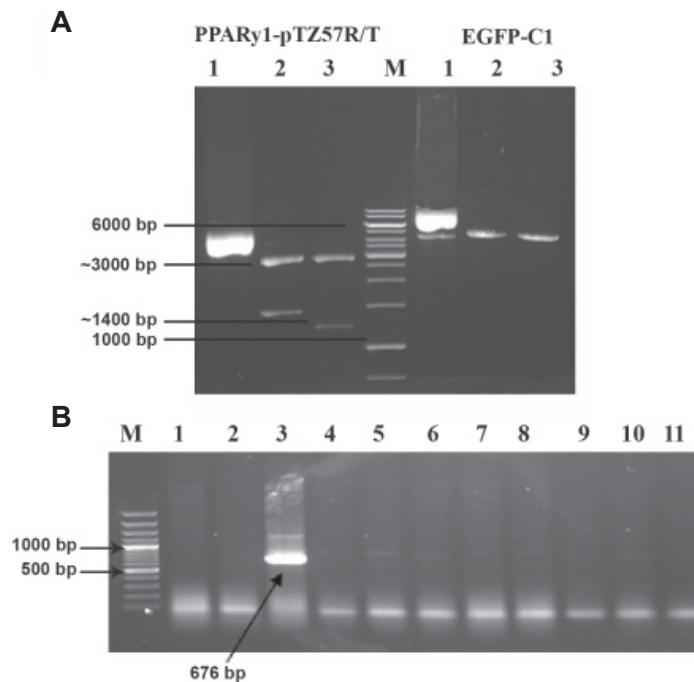


Fig 3: Enzymatic digestion of pTZ57/RT / PPAR γ 1 and pEGFP-C1 (A). Lane 1: undigested vectors, lane 2; after digestion with *Sac*I, lane 3; *Sac*I- *Kpn*I cut, M is marker (1Kbp: fermentas, Lithuania). (B) Bacterial colony insert check results for finding pEGFP-C1/PPAR γ 1.

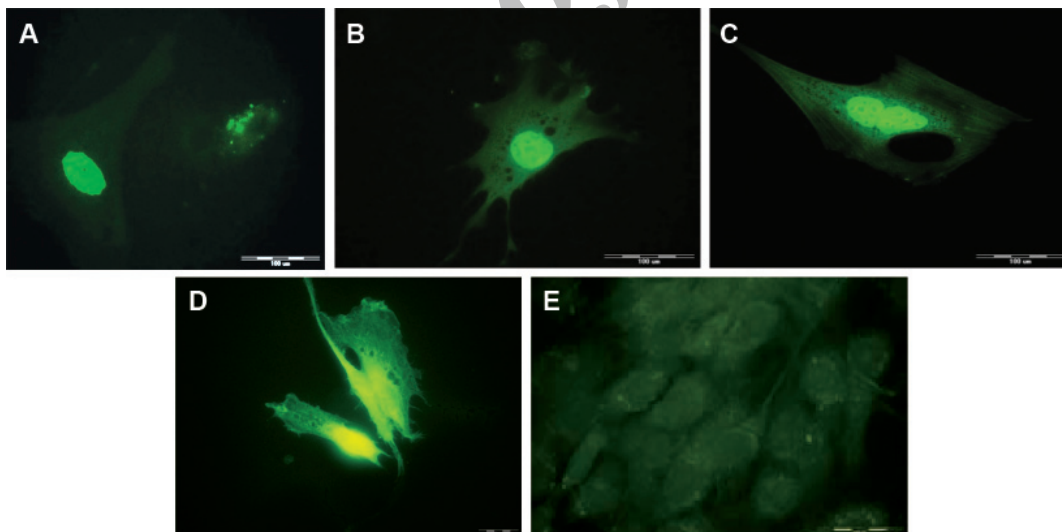


Fig 4: Transient transfection of pEGFP-C1/PPAR γ 1 into bovine fibroblast cells. A-C. Transfected cells showing nuclear and cytosolic green fluorescence of EGFP-PPAR γ 1. Magnitude \times 1000. D. Transfected cells with EGFP-C1 plasmid. EGFP is dispersed into the cytosol. E. Negative control. No fluorescence observed in the cells.

Positive colonies were identified by direct PCR approach. Sequence analysis of extracted plasmids on both strands indicated that the cDNA was 1428 bp in length with an ORF encoding a protein that consisted of 475 amino acids (Fig 2) which confirmed that the cloned cDNA was a bonafide PPAR γ 1 cDNA without mutations.

Transient transfection of pEGFP/EGFP-PPAR γ 1 cDNA in bovine fibroblast cells

The whole fragment of PPAR γ 1 cDNA was prepared by introduction into an appropriate eukaryotic expression vector (pEGFP-C1) in order to be transfected into mammalian cells. Thus, both pEGFP-C1 and pTZ57R/T/ PPAR γ 1 cDNA were treated by two enzymatic cuts as described in the

materials and methods section (Fig 3A, lanes 2 and 3). The *SacI*-*KpnI* fragment, which comprised the whole length of PPAR γ 1 cDNA was placed at the corresponding sites in pEGFP-C1 with the same coding frame and downstream of EGFP cDNA. The resultant recombinant plasmid was termed pEGFP/EGFP-PPAR γ 1. The constructed vector was extracted from several positive bacterial colonies (Fig 3B). Next, to assess intracellular localization of the PPAR γ 1 protein, transient transfection of a plasmid that expressed the EGFP-PPAR γ 1 chimeric protein was performed in bovine fibroblast cells. As green fluorescent protein (GFP) can be easily visualized under UV/blue light without any additional staining, cells were traced under UV fluorescent microscope. The bright green fluorescence was observed dominantly in the nucleus and to a lesser extent in the cytosol of transfected cells (Fig 4 A, B, C) emphasizing the main nuclear sorting of PPAR γ 1.

Discussion

The SOE approach is a fast, simple, and extremely powerful way of recombining and modifying nucleotide sequences (14). We have already used this approach to construct several truncated mutant forms of *PEP* cDNA (15). In this study, mouse PPAR γ 1 cDNA was cloned with the SOE PCR approach. This approach has currently been used for cloning of PPAR γ 1 cDNA from guinea pigs (16). PPAR γ 1 is highly expressed in mammalian adipose tissue where it plays a critical regulatory role in adipocytes (17). Thus we used adipose tissue for RNA extraction leading to cDNA cloning of PPAR γ 1. Sequence data confirmed that the cloned cDNA fragment was a bonafide PPAR γ 1 cDNA as reported earlier (18).

We have used the EGFP reporter gene to identify the intracellular localization of PPAR γ 1. GFP can be easily visualized under UV/blue light without any additional substrate or co-factor. Its assay is also non-destructive. Therefore, it has been widely used to monitor transgene expression and protein localization in a variety of cells and organisms (19). In our laboratory, EGFP have been used as a marker gene for indication of ectopic gene expression and intracellular destination of related proteins (20). Thus we have used the same technique for cloning PPAR γ 1. Using this recombinant vector which contains a chimeric form of EGFP PPAR γ 1, we will be able to chase the intracellular location of the PPAR γ 1 protein and examine its ectopic overexpression in the process of stem cell differentiation. One major concern which remains to be clarified is that recombinant protein tagging can

interfere with normal protein function or its intracellular sorting, indicating the need for verifying its efficiency (21). To examine the functionality of EGFP-PPAR γ 1 and its intracellular sorting, we have used a recombinant plasmid in this study for transfection into bovine fibroblast cells. In confirmation with previous data on mouse hepatoma and COS-1 cells (22, 23), our transfected pEGFP-PPAR γ 1 localization data clearly demonstrated predominantly nuclear and cytosolic diffused distributions in bovine fibroblast cells. Thus, concluding that PPAR γ 1 is synthesized in the cytosol and imported to the nucleus in bovine fibroblast cells, verifying the proper function of the constructed recombinant plasmid. However in 3T3-L1 preadipocytes and human peripheral blood monocytes, the high expression of PPAR γ caused punctuate and perinuclear distribution of PPAR γ (24, 25).

Conclusion

Taken together, this study has established the nuclear localization of PPAR γ 1 in bovine fibroblast cells therefore demonstrating the correct targeting activity of an exogenous PPAR γ 1. Our constructed recombinant plasmid can be used for further studies to unravel additional metabolic functions of PPAR γ since it can properly target into its destination which is the cell nucleus.

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