Original Article

Intranuclear Localization of EGFP-mouse PPARγ1 in Bovine Fibroblast Cells

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Received: 5/Apr/2009, Accepted: 1/Aug/2009

Abstract

Objective: The aim of this study was to clone PPARγ1 cDNA in an appropriate mammalian expression vector, with a chimeric cDNA form, encompassing PPARγ with enhanced green fluorescent protein (EGFP) cDNA. This recombinant plasmid will be used for further analyses to investigate the molecular mechanism of PPARγ1 for neural differentiation process. Moreover, the nuclear localization of the PPARγ1 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, PPARγ1 cDNA was synthesized and amplified to produce the entire length of ORF. RT-PCR products containing PPARγ1 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 PPARγ1 cDNA were selected for plasmid preparations and additional analysis was performed to ensure that PPARγ1 cDNA was inserted properly. Finally, to confirm the intracellular localization of EGFP-PPARγ1, bovine fibroblast cells were transfected with the recombinant plasmid.

Results: Our results from enzymatic digestion and sequencing confirmed, as expected, that PPARγ1 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: PPARγ1 cDNA was cloned and sorted into nuclear compartments of bovine fibroblast cells upon transfection.

Keywords: PPARγ, Nuclear Targeting, Enhanced Green Fluorescent protein, Cloning, Transfection

Yakhteh Medical Journal, Vol 12, No 1, Spring 2010, Pages: 97-104

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. PPARα (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, PPARγ (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 "AGGTCAXAGGTCA", (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 PPARγ. In mammals two PPARγ isoforms, PPARγ1 and PPARγ2, have been detected (12). Both isoforms are abundantly expressed in adipose tissue. PPARγ1 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). PPARγ plays a key role in adipogenesis and adipocyte gene expression, and it is the receptor for the thiazolidinedione class of insulin-sensitizing drugs (12). PPARγ exerts its function by binding to PPRE at the promoters of target genes. The mouse PPARγ gene has nine exons and extends more than 100 kilobases. Alternate transcription start sites and alternate splicing generate PPARγ1 and PPARγ2 mRNAs, which differ at their 5´-ends. Thus PPARγ1 is encoded by eight exons, whereas PPARγ2 is encoded by seven exons. The 5´-untranslated sequence of PPARγ1 is comprised of exons A1 and A2, whereas that of PPARγ2 plus the additional PPARγ2-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 PPARγ1 and γ2 (13). Due to various functions which are suggested for PPARγ, cloning of related cDNAs seems to be necessary since there is no evidence for the role of exogenous PPARγ in the process of neural cell differentiation. Thus, the aim of this study is to clone PPARγ1 cDNA in a mammalian expression vector in a chimeric cDNA type, encompassing PPARγ with enhanced green fluorescent protein (EGFP) cDNA and determine the nuclear localization of the PPARγ protein when fused to an EGFP marker.

Materials and Methods

RNA extraction

RNA was purified from the fatty tissue of a mouse (Souri 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 µl choloroform 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 centrifugatin 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 µg 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 PPARγ1 cDNA amplification was done with 2 µl 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 PPARγ1 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 PPARγ1 cDNA covering the whole length of related cDNA. The length of first fragment was 1033 bp which was amplified with primer pairs PPARγ1-F SacI and PPARγ1-R-1021 (introducing SacI restriction site at the 5' end). A second 886 bp fragment was obtained in a PCR reaction with PPARγ1-R-KpnI and PPARγ1-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 PPARγ1 cDNA was a set of PCR with the last step products as template and using primer pairs PPARγ1-F-SacI and PPARγ1-R-KpnI to produce the full length PPARγ1 cDNA (1428 bp). Moreover, in a different RT-PCR reaction with B-tubulin F and B-tubulin R primers, and with using a 2 µl 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 PPARγ1 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 PPARγ1 cDNA analyses (Bioneer, Korea). Sequence checked recombinant plasmid was digested with *SacI* (Fermentas, Lithuania) and *KpnI* (Fermentas, Lithuania). The *SacI-KpnI* fragment which contained PPARγ1 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 PPARγ1 and EGFP termed pEGFP/ EGFP- PPARγ1. 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% CO_2.

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

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 PPARγ, 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 PPARγ1 cDNA. Product sizes were as expected (Fig 2B, lanes 1 and 2). As previously described in materials and methods, the entire length of PPARγ1 cDNA (1428 bp) was produced at the second step of SOE-PCR (Fig 2C) and cloned into a pTZ57R/T vector termed pTZ57R/T/ PPARγ1 cDNA.

Table1: List of primers

PRIMER NAME		PRIMER SEQUENCE	Product length	Annealing temperatures used for PCR
Beta tubulin	F	5' - TCACTGTGCCTGAACTTACC -3'	318 bp	63°C
Beta tubulin	R	5'- GGAACATAGCCGTAAACTGC -3'		
PPARγ1-SacI	F	5'- ATTT**GAGCTC**AAGTTGACACAGAGATGCCATTCTG-3' *SacI*	1033 bp	65°C
PPARγ1-1021	R	5'-GATGGAGTCCTCATCTCAGAGG-3'		
PPARγ1-552	F	5'-GCCAACAGCTTCTCCTTCTCGGCC-3'	786 bp	60°C
PPARγ1-KpnI	R	5'- AATT**GGTACC**CTAATACAAGTCCTTGTAGATC -3' *KpnI*		
PPARγ1-SacI	F	5'- ATTT**GAGCTC**AAGTTGACACAGAGATGCCATTCTG-3' *SacI*	1450 bp	70°C
PPARγ1-KpnI	R	5'- AATT**GGTACC**CTAATACAAGTCCTTGTAGATC -3' *KpnI*		
EGFP-C1	F	5'- AACGAGAAGCGCGATCACATGC -3'	676 bp	63°C
PPARγ1-575*	R	5'- GGCCGAGAAGGAGAAGCTGTTGGC -3'		

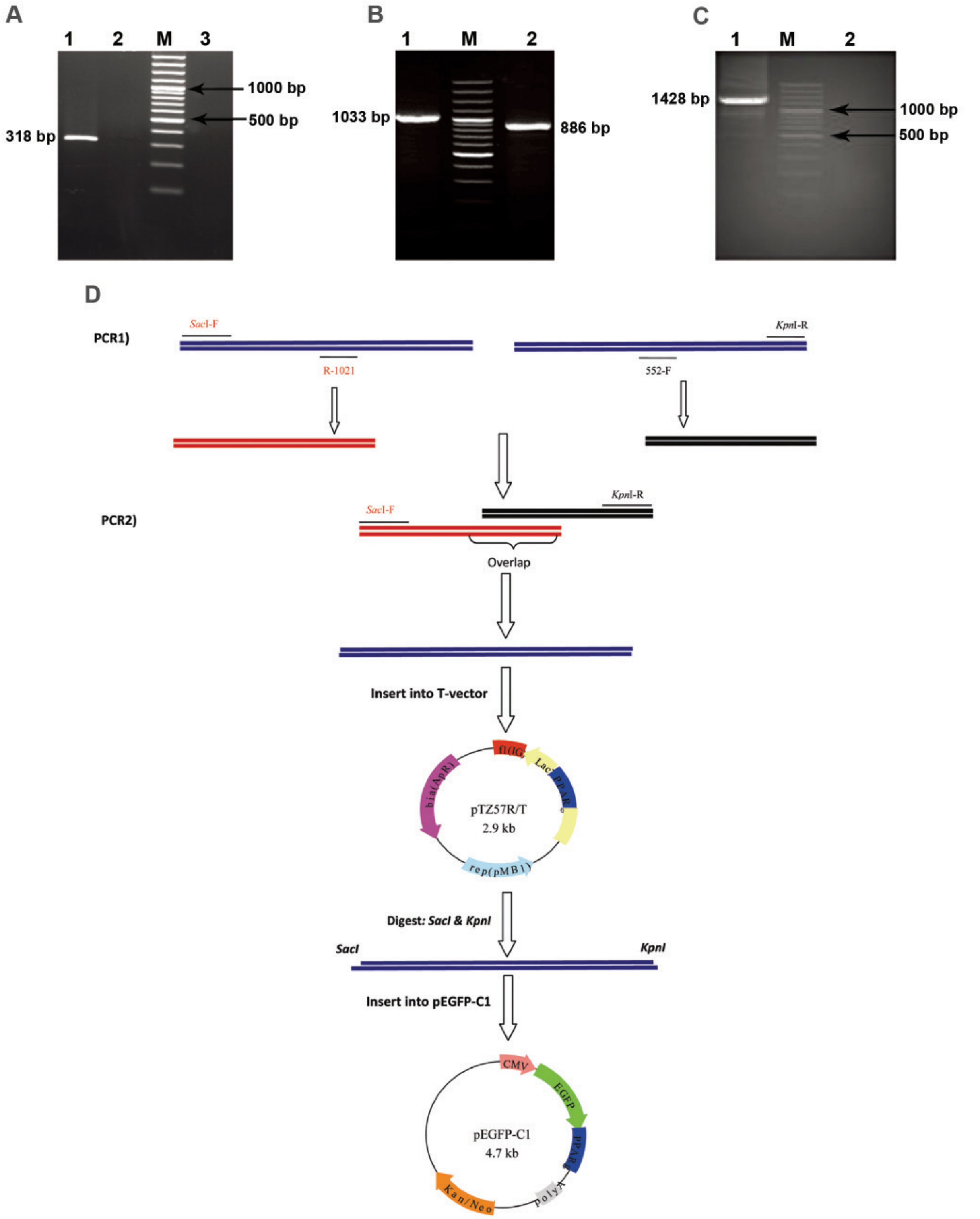

Fig 1: RT-PCR products of b-tubulin and PPARγ1 fragments. A. Partial fragment of amplified b-tubulin cDNA (lane 1), lane 2 is the control sample, lane 3 is blank; M is the marker (100 bp: Fermentas, Lithuania). B. Two amplified fragments of PPARγ1 cDNA (lanes 1, 2); M is the marker (100 bp: Fermentas, Lithuania). C. The entire amplified PPARγ1 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 PPARγ1 cDNA.

A

atggttgacacagagatgccattctggcccaccaacttcggaatcagctctgtggacctc
tccgtgatggaagaccactcgcattcctttgacatcaagcccttttaccacagttgatttc
tccagcatttctgctccacactatgaagacattccattcacaagagctgacccaatggtt
gctgattacaaatatgacctgaagctccaagaataccaaagtgcgatcaaagtagaacct
gcatctccaccttattattctgaaaagacccagctctacaacaggcctcatgaagaacct
tctaactccctcatggccattgagtgccgagtctgtggggataaagcatcaggcttccac
tatggagttcatgcttgtgaaggatgcaagggtttttccgaagaaccatccgattgaag
cttatttatgataggtgtgatcttaactgccggatccacaaaaaaagtagaaataaatgt
cagtactgtcggtttcagaagtgccttgctgtggggatgtctcacaatgccatcaggttt
gggcggatgccacaggccgagaaggagaagctgttggcggagatctccagtgatatcgac
cagctgaacccagagtctgctgatctgcgagccctggcaaagcatttgtatgactcatac
ataaagtccttcccgctgaccaaagccaaggcgagggcgatcttgacaggaaagacaacg
gacaaatcaccatttgtcatctacgacatgaattccttaatgatgggagaagataaaatc
aagttcaaacatatcacccccctgcaggagcagagcaaagaggtggccatccgaattttt
caagggtgccagtttcgatccgtagaagccgtgcaagagatcacagagtatgccaaaaat
atccctggtttcattaaccttgatttgaatgaccaagtgactctgctcaagtatggtgtc
catgagatcatctacacgatgctggcctccctgatgaataaagatggagtcctcatctca
gagggccaaggattcatgaccagggagttcctcaaaagcctgcggaagccctttggtgac
tttatggagcctaagtttgagtttgctgtgaagttcaatgcactggaattagatgacagt
gacttggctatatttatagctgtcattattctcagtggagaccgcccaggcttgctgaac
gtgaagcccatcgaggacatccaagacaacctgctgcaggccctggaactgcagctcaag
ctgaatcacccagagtcctctcagctgttcgccaaggtgctccagaagatgacagacctc
aggcagatcgtcacagagcacgtgcagctactgcatgtgatcaagaagacagagacagac
atgagccttcacccccctgctccaggagatctacaaggacttgtattag

B

MVDTEMPFWPTNFGISSVDLSVMEDHSHSFDIKPFTTVDFSSIS

APHYEDIPFTRADPMVADYKYDLKLQEYQSAIKVEPASPPYYSEKTQLYNRPHEEPSN

SLMAIECRVCGDKASGFHYGVHACEGCKGFFRRTIRLKLIYDRCDLNCRIHKKSRNKC

QYCRFQKCLAVGMSHNAIRFGRMPQAEKEKLLAEISSDIDQLNPESADLRALAKHLYD

SYIKSFPLTKAKARAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQEQSKEVA

IRIFQGCQFRSVEAVQEITEYAKNIPGFINLDLNDQVTLLKYGVHEIIYTMLASLMNK

DGVLISEGQGFMTREFLKSLRKPFGDFMEPKFEFAVKFNALELDDSDLAIFIAVIILS

GDRPGLLNVKPIEDIQDNLLQALELQLKLNHPESSQLFAKVLQKMTDLRQIVTEHVQL
LHVIKKTETDMSLHPLLQEIYKDLY

C

Fig 2: Schematic representation of PPARγ1 cDNA and protein sequences. A. ORF sequence of PPARγ1 cDNA. B. Produced amino acid residues of PPARγ1 cDNA. C. Partial sequence of PPARγ1 cDNA representing the 5'-part of PPARγ1 cDNA and SacI restriction site. D. Partial sequence of PPARγ1 cDNA representing the 3'-part of PPARγ1 cDNA and KpnI restriction site.

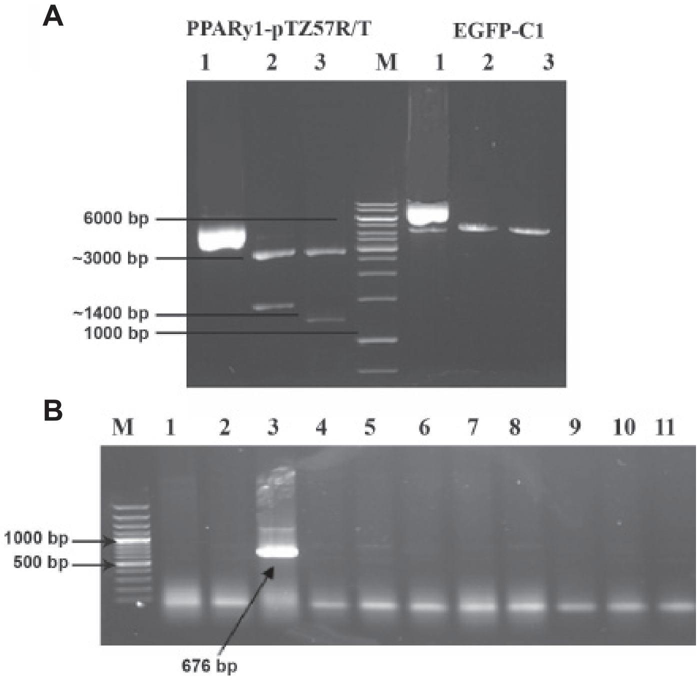

Fig 3: Enzymatic digestion of pTZ57/RT / PPARγ1 and pEGFP-C1 (A). Lane 1: undigested vectors, lane 2; after digestion with SacI, lane 3; SacI- KpnI cut, M is marker (1Kbp: fermentas, Lithuania). (B) Bacterial colony insert check results for finding pEGFP-C1/PPARγ1.

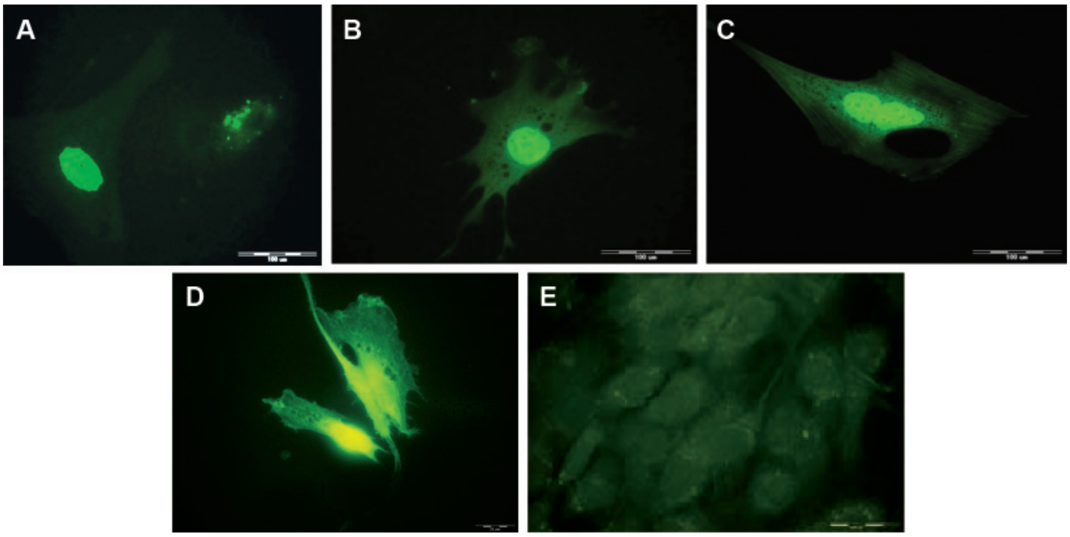

Fig 4: Transient transfection of pEGFP-C1/PPARγ1 into bovine fibroblast cells. A-C. Transfected cells showing nuclear and cytosolic green fluorescence of EGFP-PPARγ1. Magnitude × 1000. D. Transfected cells with EGFP-C1 plasmid. EGFP is dispersed into the cytosol. E. Negative control. No fluorescence 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 PPARγ1 cDNA without mutations.

Transient transfection of pEGFP/EGFP-PPARγ1 cDNA in bovine fibroblast cells

The whole fragment of PPARγ1 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/ PPARγ1 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 PPARγ1 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-PPARγ1. The constructed vector was extracted from several positive bacterial colonies (Fig 3B). Next, to assess intracellular localization of the PPARγ1 protein, transient transfection of a plasmid that expressed the EGFP-PPARγ1 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 PPARγ1.

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 PPARγ1 cDNA was cloned with the SOE PCR approach. This approach has currently been used for cloning of PPARγ1 cDNA from guinea pigs (16). PPARγ1 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 PPARγ1. Sequence data confirmed that the cloned cDNA fragment was a bonafide PPARγ1 cDNA as reported earlier (18).

We have used the EGFP reporter gene to identify the intracellular localization of PPARγ1. 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 PPARγ1. Using this recombinant vector which contains a chimeric form of EGFP PPARγ1, we will be able to chase the intracellular location of the PPARγ1 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- PPARγ1 and its intraclleular 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-PPARγ1 localization data clearly demonstrated predominantly nuclear and cytosolic diffused distributions in bovine fibroblast cells. Thus, concluding that PPARγ1 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 PPARγ caused punctuate and perinuclear distribution of PPARγ (24, 25).

Conclusion

Taken together, this study has established the nuclear localization of PPARγ1 in bovine fibroblast cells therefore demonstrating the correct targeting activity of an exogenous PPARγ1. Our constructed recombinant plasmid can be used for further studies to unravel additional metabolic functions of PPARγ since it can properly target into its destination which is the cell nucleus.

Acknowledgments

This study was supported by a grant awarded to Dr. K. Ghaedi from the Royan Institute, Isfahan, Iran (project No.148-5). There is no conflict of interest in this study.

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