Molecular Vision 4: 7, 1998 <http://www.emory.edu/molvis/v4/p7>
Received 9 January 1998 | Accepted 12 March 1998 | Published 20 April 1998

Structural and Comparative Analysis of the Mouse Gene for Pigment Epithelium-Derived Factor (PEDF)

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Purpose: Pigment epithelium-derived factor (PEDF), a neurotrophic protein, is a member of the serine protease inhibitor supergene family. PEDF promotes both neuronal phenotype in cultured Y79 cells and neuronal survival in cultured cerebellar granulocytes. The purpose of this study was to clone the mouse PEDF gene and to determine its structure and levels of expression in different tissues.

Methods: The mouse PEDF cDNA was cloned from a mouse liver cDNA library using human PEDF cDNA as a probe. The mouse PEDF gene was cloned from a mouse ES genomic P1 library. DNA sequencing was performed using a PE-Applied Biosystems model 373 automated fluorescent sequencer.

Results: The mouse PEDF cDNA is 1461 bp in length and contains an open reading frame of 417 amino acids. The mouse PEDF gene spans approximately 13 kb and, like the human, it is fragmented into 8 exons. The splice sites follow the AG/GT consensus rule. Southern blot analysis indicates that the mouse genome contains only one gene for PEDF. Northern blot analysis shows the presence of the PEDF transcript in a broad range of adult mouse tissues with liver showing the highest level of expression.

Conclusions: The mouse and human PEDF promoters share overall 27% similarity but are nearly identical between mouse +86 to -166 and human +102 to -96. The present study will allow us to move from in vitro experiments to in vivo studies through the development of a “knock-out” mouse model.

Pigment epithelium-derived factor (PEDF) is a 50 kDa neurotrophic protein first demonstrated in conditioned medium of cultured human fetal retinal pigment epithelial (RPE) cells [1]. PEDF mRNA is detected in the eye of the human fetus at approximately 17 weeks of gestation [2]. PEDF also is present in the adult RPE of other species such as monkey, cow and chicken as well as in most other non-retinal adult tissues. PEDF is secreted into the interphotoreceptor matrix of the retina, and may play a role in retinal differentiation. PEDF induces neuronal differentiation of cultured human Y-79 retinoblastoma cells in vitro [1]. Differentiation is both morphological as well as biochemical. Specifically, morphologically undifferentiated Y-79 cells extend long, neurite-like processes in response to PEDF [3]. Increased expression of neuronal marker molecules occurs coincident with the morphological changes [3]. Additionally, PEDF is implicated in cell cycle and aging [2,3]. Furthermore, PEDF markedly enhances the survival of neurons in culture [4].

PEDF cDNA was cloned from a human fetal eye cDNA library and its derived amino acid sequence identified it as a member of the serine protease inhibitor (serpin) supergene family [5]. However, PEDF behaves as a non-inhibitory serpin in that its neurotrophic activity does not require the serpin reactive loop [6]. PEDF is also of interest since its gene was localized to human chromosome 17p [7], a region to which a number of hereditary diseases map. Recently, it was shown that the PEDF gene is tightly linked to the RP13 locus on chromosome 17p13.3 [8]. Based on these facts, we thought it of interest to characterize the mouse PEDF gene. The mouse PEDF cDNA was recently cloned by another group [9] while searching for secreted proteins. Here we present a more detailed analysis of the mouse PEDF cDNA, gene structure, and promoter sequence as well as quantitative expression in a number of mouse tissues.

METHODS

Isolation of P1 clones— The P1 clones were isolated from a mouse ES genomic P1 library using mouse-specific primers 5989 (5'-TGGCAAACCCGTGAAGCTCA-3') and 5990 (5'-GAGGCTACACTGAAGCTACC-3') by Genome Systems Inc. (St. Louis, MO).(Here and throughout, oligonucleotide numbers are internal references and do not denote positioning).

Oligonucleotides— Oligonucleotide primers were synthesized using a DNA synthesizer, Model 392, from Applied Biosystems Inc.(Foster City, CA). The oligonucleotides were deprotected at 55 °C for 12 h, lyophilized and used without further purification. The sequence and location of oligos used to study the introns are given in Table 1. The following additional primers were used to amplify PCR products for exon 5, exon 1, and the promoter region from our mouse P1 (p11145) clone.

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6069 5'-CCGGGCTCTCTACTACGACCTGAT-3'
6700 5'-AGCCAGGGAGCGTCTTCCATAT-3'
T3 5'-AATTAACCCTCACTAAAGGG-3'

Other oligos used for sequencing are not listed.

Polymerase chain reaction (PCR)— The PCR amplifications were performed for 30 cycles using standard protocols. The annealing and extension times were varied depending on the oligonucleotide composition or length of the product amplified, respectively. PCR products were purified using Wizard™ PCR Preps DNA purification system (Promega, Madison, WI) following the manufacturer's protocol.

Screening of mouse and bovine liver cDNA libraries— Mouse and bovine liver Lambda ZAP cDNA libraries (Stratagene, La Jolla, CA) were screened using a standard protocol [14]. Hybridization of the colony lift was performed at 42 °C for 18 h in Hybrisol™ (Oncore, Gaithersburg, MD) and approximately 10^6 cpm/µl of a ^{32}P-labeled human PEDF probe [5]. Positive clones were isolated, excised, and plasmid DNA purified using a Qiagen Plasmid Purification Kit following the manufacturer's protocol (Qiagen Inc., Chatsworth, CA).

Automated Fluorescence sequencing— A PE-Applied Biosystem automated fluorescence sequencer (373A) was used to perform the DNA sequencing (Applied Biosystems, Foster City, CA). The ABI PRISM™ FS Dye terminator cycle sequencing kit was used following the manufacturer's protocol. Sequencing reaction products were purified using STE Select-D G-50 columns (5 Prime -> 3 Prime Inc., Boulder, CO).

Preparation of PCR probe for library screening, Southern and northern blots— Human PEDF cDNA (*Eco* RI/*Hind* III fragment) was labeled with [^{32}P] dCTP (Amersham, Arlington Heights, IL) to a specific-activity of approximately 5 x 10^9 cpm/µg using RTS RadPrime DNA labeling system (Life Technologies, Gaithersburg, MD). This probe was used for screening the cDNA libraries. The insert from the mouse pMou12A cDNA clone was used for probing the Southern and northern blots.

RNA extraction and northern blot analysis— Total RNA was isolated from liver and retina of NIH albino mice using RNAazol (Cinna/Biotec Laboratory, Friendswood, TX). Other RNA samples were purchased from Clontech (Palo Alto, CA). Samples containing total RNA (20 µg) were separated by electrophoresis in a 1% agarose formaldehyde gel at 25 V for 3 h. Following electrophoresis, the gel was stained with SYB Green II (Molecular Probes, Eugene, OR) then scanned in the Storm 860 instrument (Molecular Dynamics, Sunnyvale, CA). An estimation of the hybridizing band size was obtained by comparison with the migration of RNA molecular weight standards (Life Technologies). The blots were analyzed and quantitated using ImageQuant Software (Molecular Dynamics).

TABLE 1. OLIGONUCLEOTIDES USED TO DETERMINE THE INTRON SIZE OF THE MOUSE PEDF GENE.

Oligonucleotide Number	Sequences 5' -> 3'	Location	PCR Product
6253	CGCAGTGAGAGAAGCTGCCG	Exon 1->	
6176	CAGAGGAGTAGCACCAGGGC	Exon 2<-	4000 bp
6251	GCCCTGGTGCTACTCCTCTG	Exon 2->	
6177	TGCTGCCAGCTTGTTCACA	Exon 3<-	650 bp
6252	TGTGAACAAGCTGGCAGCA	Exon 3->	
6063	ATCAGGTCGTAGTAGAGAGCCCGG	Exon 4<-	750 bp
6017	ACCTGATCACCAACCTGACAT	Exon 4->	
6468	GTCCCATAGGACTTCTCCAG	Exon 5<-	650 bp
6465	TGCCCAGTGCCCTCAGCAT	Exon 5->	
6073	CCAAGCCGTATCGTAAGATG	Exon 6<-	1050 bp
6255	CATCTTACGATACGGCTTGG	Exon 6->	
6482	AAGTTCTGGGTCACGGTCAG	Exon 7<-	3000 bp
6467	GACTATCCAAGCTGTGCTGA	Exon 7->	
6257	TGAGCTTCACGGGTTTGCCA	Exon 8<-	600 bp

The arrows indicate 5'-> or 3'<- orientation.

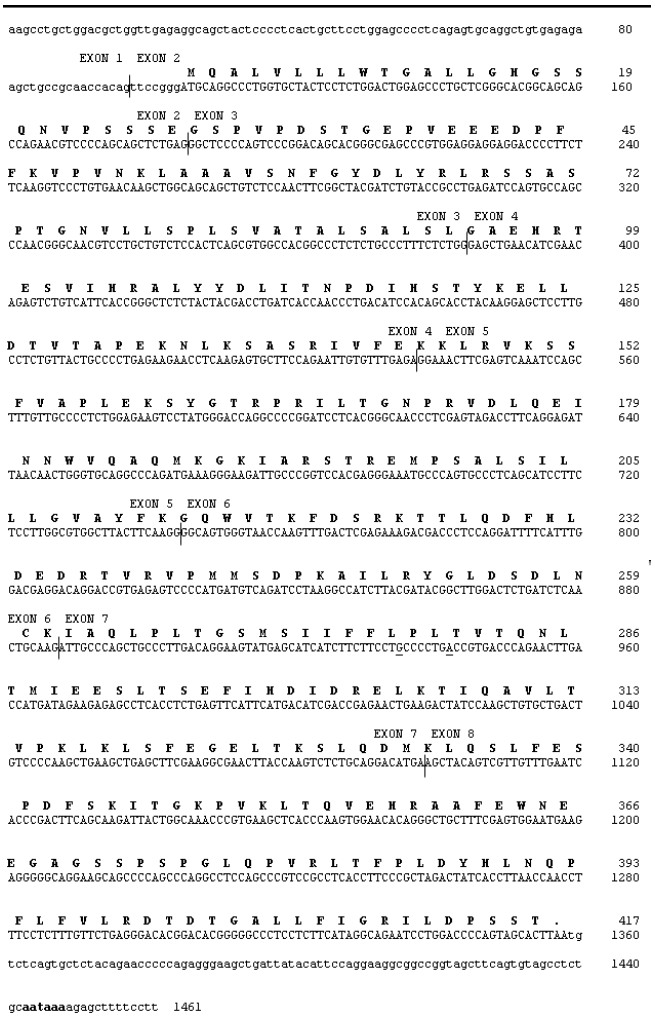

Figure 1. Full length sequence of the mouse PEDF cDNA. Sequence was obtained using a mouse liver cDNA clone (pMou 13). The exons are labeled and separated by a line. The untranslated sequences are shown in lower case and the coding regions in upper case letters. The deduced amino acids are shown in single letter code above the second base of the codon. The poly (A) signal, **aataaa**, is shown in bold. The underlined sequence, GGCAG (673-677), was absent in some of the clones (e.g. pMou 12A) isolated. The GenBank accession number for mouse PEDF cDNA is AF017057.

Screening of mouse liver genomic library— NIH3T3 mouse liver genomic library in the Lambda FIX II vector (Stratagene) was screened as described above for cDNA libraries. The screening of the mouse genomic ES P1 library was performed by Genome Systems Inc. (St. Louis, MO) [10] using oligonucleotides 5989 (5'-TGGCAAACC-CGTGAAGCTCA-3') and 5990 (5'-GAGGCTACACTG-AAGCTACC-3') which were designed from the mouse PEDF cDNA sequence. Three clones were isolated: p11143, p11144 and p11145. The P1 clone, p11145, contained the entire PEDF gene and flanking sequences.

Southern blot analysis— P1 clones were grown in Super Broth (Quality Biologicals, Gaithersburg, MD) with 25 µg/ml of kanamycin (Life Technologies). Mouse genomic DNA and p11145 DNA were subjected to digestion with restriction enzymes. The digested DNA was separated on 1% agarose TBE gel using a CHEF DRII pulse field apparatus (Bio-Rad Laboratories, Inc., Hercule, CA) set for 10 h at 200 v and 2 s pulses. The gel was transferred to the membrane, probed, and autoradiographed as described above for northern blot.

Subcloning of the mouse PEDF gene fragment and sequencing— P1 clone, p11145 as well as BlueScript plasmid (Stratagene) were digested separately with *Bam* HI, *Hind* III and *Eco* RI (Life Technologies). Digested P1 fragments of particular restriction enzyme were subcloned into BlueScript plasmid digested with the same restriction enzyme. JM 109 cells were transformed and positive clones were selected by hybridization with the PEDF cDNA probe as described above. Plasmid preparations of these clones were used for sequencing intron-exon junctions and intron size was determined by PCR using oligos given in Table 1. Intron size was further confirmed by amplification of the P1 clone and sequencing.

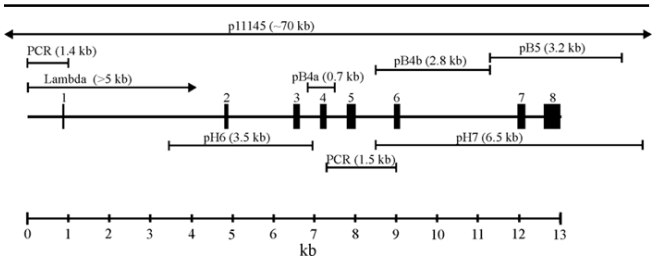

Figure 2. Scale map of the mouse PEDF gene. The map shows the exons (solid rectangles; 1-8) along the gene. The GenBank accession numbers for mouse PEDF promoter and various exons are: promoter and exon 1 - AF017050, exon 2 - AF017051, exon 3 - AF017052, exons 4 & 5 - AF017053, exon 6 - AF017054, and exons 7 & 8 - AF017055.

RESULTS

Isolation of mouse and bovine PEDF cDNAs— We isolated one bovine and two mouse cDNA clones from the appropriate liver cDNA libraries. The mouse PEDF cDNA was recently cloned [9] (GenBank Accession number D50460). The two mouse clones (pMou12A and pMou13) and the one bovine clone (pBov13) we isolated were completely sequenced in both directions. The sequence of the mouse PEDF cDNA was not given in reference 9. We now show this sequence with the appropriate information in Figure 1. The complete open reading frame for mouse PEDF is 1254 bp and codes for a peptide 417 amino acids in length. The mouse PEDF cDNA contains 103 bases of 3' untranslated region and a poly (A) signal at position 1443. The sequences for mouse and bovine cDNA have been placed in GenBank with accession numbers AF017057 and AF017058, respectively.

Our mouse PEDF sequence was different from the sequence in GenBank (Accession number D50460) in two bases at positions 935 (G to C), and 942 (A to G). The change at position 942 alters the amino acid at position 280 from a threonine to an alanine.

Cloning and Sequencing of the Mouse PEDF Gene— The mouse PEDF gene was isolated by PCR screening of a mouse genomic P1 library using oligonucleotides specific to mouse

Table 2. Intron-exon Junctions in Mouse PEDF Gene.

Exon #	Size (bp)	Exon	Donor	Intron Size (bp)	Acceptor	Exon	Exon#
				PROMOTER	tgtaatctg	AAGCCTGCT	1
1	97	CAACCACAG	gtaaggcag	…~3900	ctcttgtag	TTCCGGGAT	2
2	88	AGCTCTGAG	gtcagtagg	…~1500	ctaacccag	GGCTCCCCA	3
3	199	TTTCTCTGG	gtgagtgtc	…~ 500	ctgtggcag	GAGCTGAAC	4
4	156	TTGAGAGGA	gtcagtagc	…450…	cctctcaag	AACTTCGAG	5
5	204	ACTTCAAGG	gtgagggct	…~ 900	tccttccag	GGCAGTGGG	6
6	143	AACTGCAAG	gtctgtagg	…~2900	ttgggttag	ATTGCCCAG	7
7	211	AGGACATGA	gtatgtttg	…438…	tctccacag	AGCTACAGT	8
8	363	TTTCCTTAA	tgtttgctt				

cDNA as described above. One of the P1 clones, p11145, contained the entire PEDF gene and was used to determine its structure (Figure 2). The p11145 was digested with *Bam*HI and *Hind*III separately and the fragments were subcloned into pBlueScript KS$^+$. Three *Bam*HI subclones were obtained: pB4a (0.7 kb), which contained exon 4; pB4b (2.8 kb), which contained exon 6; and pB5 (3.2 kb), which contained exons 7 and 8. In addition, two *Hind*III subclones were obtained: pH6 (3.5 kb), containing exons 2 and 3; and pH7 (6.5 kb), which contained exons 6, 7, and 8. The plasmid subclones were then sequenced in both directions to determine the intron-exon junctions of exons 2, 3, 4, 6, 7, and 8. The sequence of exon 5 was obtained from a PCR product of 1.5 kb fragment generated with primer 6069 (in exon 4) and primer 6073 (in exon 6). The exon 1 and intron 1 junction was obtained by direct sequence of a Lambda genomic clone (λ1) and from sequencing of a PCR product of 3.9 kb generated from p11145 with primers 6253 and 6176 (Table 1). The sequence of exon 1 and the promoter were obtained from a subcloned PCR product of 1.4 kb generated from the λ1 clone using primers T3 and 6700 (in intron 1).

The mouse PEDF gene spans approximately 13 kb and is divided into 8 exons and 7 introns (Figure 2). All intron-exon junctions were confirmed by at least two independent sequencing reactions on each strand. The intron-exon junctions and their flanking sequences are shown in Table 2. All intron-exon junctions conform to the AG/GT consensus rule.

Comparison of the Mouse and Human PEDF promoter sequences— The mouse and human PEDF promoters were compared to assess the conserved regions that may be important in the transcriptional regulation of the gene. The mouse and human PEDF promoters share low similarity overall but are nearly identical between mouse +86 to -166 and human +102 to -96 (Figure 3). The human PEDF promoter contains two *Alu* repetitive sequences (underlined) which are not conserved between mouse and human. The mouse promoter contains a $(CT)_{20}$ repeat between -578 and -618 and a $(GT)_{19}$ repeat between -869 and -907 that are not conserved in the human sequence. However, the two mouse repeat regions align roughly with the two *Alu* repetitive sequences. Overall, there is only 27% sequence identity between mouse and human promoters in the roughly 1500 bps sequence aligned.

Southern blot analysis— In order to determine the complexity of the mouse PEDF gene, the genomic P1 clone p11145 and mouse genomic DNA were digested with *Bam*HI. The restricted DNAs were separated by pulse-field gel electrophoresis, blotted, and probed with the mouse cDNA insert from pMou 13 clone. Both the digested genomic DNA as well as p11145 show two major bands at ~4.0 kb and 3.2 kb suggesting that the mouse PEDF gene, like its human counterpart, is a single gene (Figure 4).

Northern blot analysis— Northern blot analysis was performed on several mouse tissues. The SYB green II stained gel and the respective probed blot were scanned using the Storm 860 instrument. The relative levels of expression were normalized to the ribosomal 18S band using Molecular Dynamics Image Quant software (Figure 5). The PEDF mRNA is detectable in most of the mouse tissues tested but there is a wide range of variation. The message is most abundant in liver but is barely detectable in retina.

DISCUSSION

Accumulating data indicate that PEDF has interesting

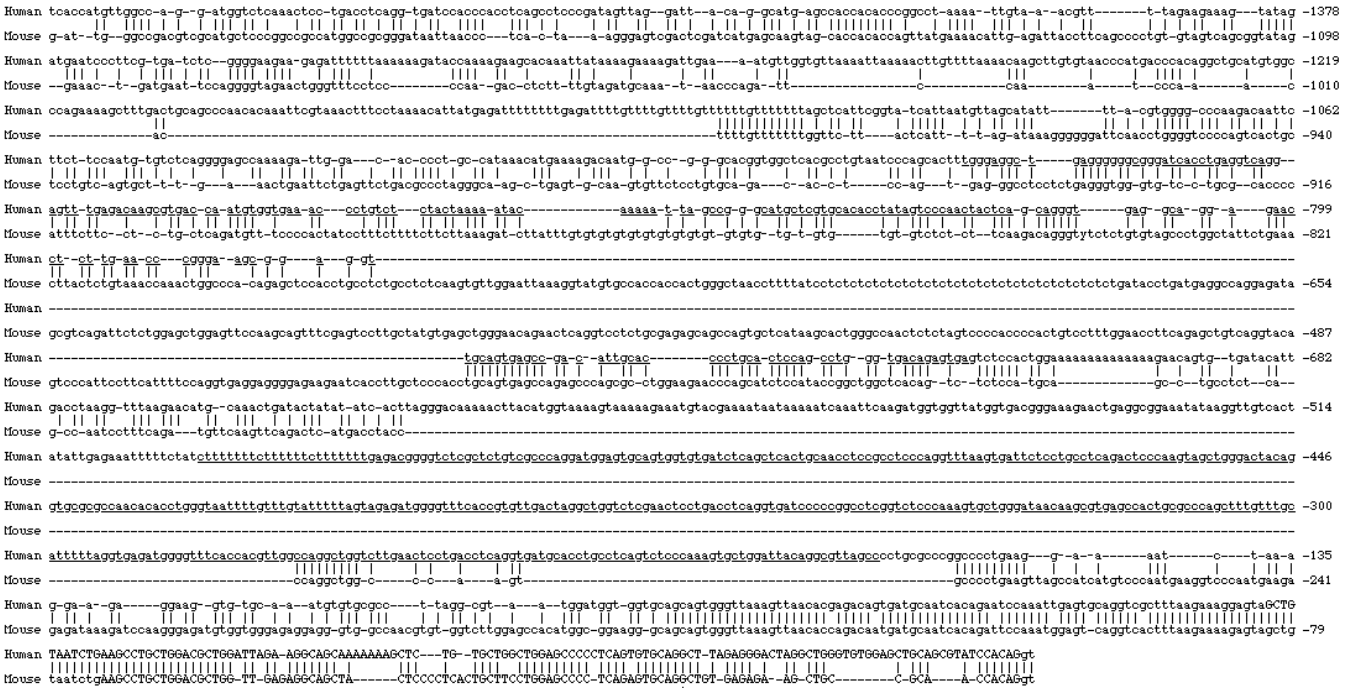

Figure 3. Sequence analysis of mouse PEDF putative promoter region and comparison with human. Exon 1 is shown in upper case letters. Human *Alu* sequences are underlined.

biological effects on neurons and glial cells. It may be involved in cell cycle events and senescence. Specifically, in vitro studies demonstrate that it has potent neuronal differentiation [1] as well as neuronal survival activity [4] in cultured cerebellar granule cells. PEDF also inhibits proliferation of glial cells in culture [4], making this a particularly interesting neurotrophic candidate for use in neuronal degenerative diseases. More generally, it has been shown that PEDF is expressed in a cell-cycle specific manner in cultured WI-38 fibroblast cells and disappears at the onset of senescence [3]. Similar evidence has also been obtained in aging RPE cells in culture [2]. Taniwaki et al. investigated the ability of PEDF to protect against glutamate neurotoxicity [12]. PEDF significantly reduced the glutamate-induced neuronal death of cerebellar granule cells in mice. These properties now need to be studied in an in vivo biological system, especially the factors that control PEDF activity at the gene level. The mouse affords such a model.

The mouse PEDF peptide is well conserved between the three species compared. While the overall similarity is only 85.2% and 83.2% to human and bovine, respectively, most of the differences are in the N-terminus between amino acids 20-40, after the lead peptide sequence.

The mouse PEDF gene structure is similar to the human (Figure 3). The gene spans ~13 kb and is fragmented into 8 exons. The 5' and 3' untranslated regions are shorter in the mouse than in the human. The transcription start site seems to be approximately 11 bases further downstream in the mouse than in the human gene, although this has not been confirmed. The translation start site is in the same position in exon 2. The mouse and human promoters are nearly identical between mouse +86 to -166 and human +102 to -96 (Figure 3). This suggests that most of the important elements in the transcriptional regulation of the PEDF gene are in the proximal promoter and exon 1, although a number of smaller matching sequences can be observed upstream. The *Alu* repetitive sequences observed in the human promoter [12] are absent from the mouse gene. The mouse instead contains CT and GT repeats in roughly the same area. Northern blot analysis suggests that the tissue specificity of PEDF mRNA expression is similar between mouse and human, but the levels of expression are markedly different. Human northern blots published by Tombran-Tink et al. [12], although not quantitated, indicate high expression in liver as well as a number of other tissues including testes, heart and lungs [12]. Mouse expresses PEDF in those tissues but liver PEDF content is roughly five-fold more than lung and heart and ten-fold more than brain and testes. In humans, the expression between brain and testis is markedly different while in mouse they are roughly the same. Another major difference is in the retina. Human retina shows detectable levels of PEDF while it is essentially

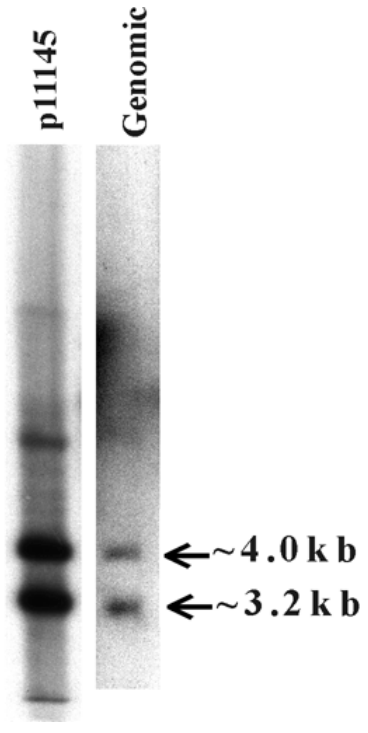

Figure 4. Southern analysis of genomic P1 clone (p11145) and genomic DNA after restriction with *Bam*HI. Restriction fragments derived from P1 clone p11145 and genomic DNA were run on a 1% agarose gel in a pulse field apparatus as described in Methods. The gel was blotted and transferred using standard conditions. The blot was hybridized with the ^{32}P-labeled mouse PEDF cDNA probe.

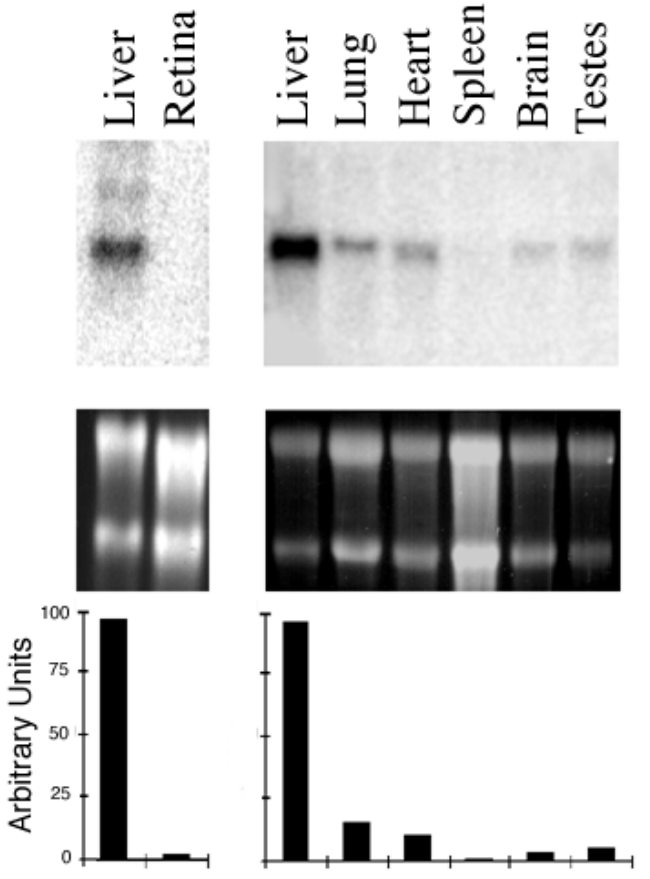

Figure 5. Multitissue Northern blot analysis of mouse PEDF mRNA. The northern blot was performed as described in Methods. The upper panel shows the autoradiogram of the probed blot, the middle panel is the SYB green II stained gel, and the lower panel shows the relative PEDF mRNA levels normalized to the ribosomal 18S band.

undetectable by northern blot in mouse. The levels of expression in mouse tissues reported by Shirozu et al. [9] are similar to those we observed (Figure 5).

The present study thus gives important information on the mouse PEDF cDNA, gene structure, and expression. Comparative promoter analysis also uncovered an area of similar sequence in the mouse and human genes indicating a possible area of importance in controlling PEDF gene transcription. This information now allows us to move from in vitro experiments to in vivo studies to determine the biological role(s) of PEDF through a "knock-out" mice animal model.

ACKNOWLEDGEMENTS

We thank Dr T. Schoen for his help in preparation of RNA samples.

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