

Short Communication

Genomic Organization, Expression Analysis, and Chromosomal Localization of the Mouse *PEX3* Gene Encoding a Peroxisomal Assembly Protein

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The peroxin Pex3p has been identified as an integral peroxisomal membrane protein in yeast where *pex3* mutants lack peroxisomal remnant structures. Although not proven in higher organisms, a role of this gene in the early peroxisome biogenesis is suggested. We report here the cDNA cloning and the genomic structure of the mouse *PEX3* gene. The 2 kb cDNA encodes a polypeptide of 372 amino acids (42 kDa). The gene spans a region of 30 kb, contains 12 exons and 11 introns and is located on band A of chromosome 10. The putative promoter region exhibits characteristic housekeeping features. *PEX3* expression was identified in all tissues analyzed, with the strongest signals in liver and in testis, and could not be induced by fenofibrate. The data presented may be useful for the generation of a mouse model defective in *PEX3* in order to clarify the yet unknown functional impact of disturbances in early peroxisomal membrane assembly.

Key words: Chromosomal localization / Fenofibrate induction / Peroxin / Peroxisomal biogenesis / Peroxisomal ghost / Tissue expression.

Peroxisome biogenesis includes peroxisome proliferation, membrane biogenesis and peroxisomal matrix protein import (Subramani *et al.*, 1997). Proteins involved in

the assembly of peroxisomes are encoded by *PEX* genes and are termed peroxins (Distel *et al.*, 1996). While at least 23 peroxins have been reported to be essential for peroxisome biogenesis, the exact functions and interactions of only a limited number of these proteins have been determined so far. Data from yeast suggested that the *PEX3* gene codes for an integral membrane protein essential for both peroxisomal membrane assembly and membrane protein import (Höhfeld *et al.*, 1991; Snyder *et al.*, 1999b). In a previous study we cloned and characterized the human *PEX3* cDNA (Kammerer *et al.*, 1998). Pex3p has been shown to interact with Pex19p in yeast (Götte *et al.*, 1998; Snyder *et al.*, 1999a), and in humans (Soukupova *et al.*, 1999). *Saccharomyces cerevisiae* cells deficient in either Pex3p or Pex19p were reported to lack peroxisomal remnant structures, also called peroxisomal ghosts (Götte *et al.*, 1998). These observations and the recent identification of multimeric peroxisomal assembly protein complexes are consistent with the hypothesis that these two proteins are essential players in peroxisomal membrane biogenesis and peroxisomal membrane protein localization (Snyder *et al.*, 1999b). However, the precise contribution of these peroxins to the maturation of early peroxisomes and human disease pathogenesis is not yet known. The generation of mice with targeted disruption of early peroxin genes, such as *PEX3*, is expected to deliver more information about biological relevance and function. We report here the cDNA sequence and expression data as well as the analysis of the genomic structure and the chromosomal localization of the mouse *PEX3* gene.

The expressed sequence tag (EST) database dbEST (Boguski *et al.*, 1993) was probed with the human *PEX3* cDNA sequence. Four mouse EST clones, m1 (IMAGp998L081096), m2 (IMAGp998H081406), m3 (IMAGp998P072169), and m4 (IMAGp998M232137) were obtained from the Resource Center of the German Human Genome Project (Lehrach *et al.*, 1990). The longest clone, m1, was completely sequenced and revealed to contain 1511 bp including the 3' portion of the gene. The cDNA was completed by 5' RACE-PCR (Marathon-Ready mouse liver cDNA, Clontech) and showed the following features: a 5' untranslated region of 167 bp followed by an open reading frame of 1116 bp (372 amino acids) and a 3' untranslated sequence of 729 bp. A consensus poly(A) signal was identified 34 bp upstream of the poly(A) at position +2017 relative to the transcription start site. The usage of an alternative poly(A) site in the 3' untranslated

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region was confirmed by 3' RACE-PCR. Two further poly(A) signals at positions +1341 and +1376 were detected in several clones followed by a poly(A) tail 16–35 bp downstream. The position of the two alternative poly(A) sites at about 70 and 730 bp downstream of the TGA stop codon is in agreement with the data on the human *PEX3* gene (Kammerer *et al.*, 1998). Using the ClustalW program (Thompson *et al.*, 1994) from the Baylor College of Medicine, we performed a pairwise alignment of the deduced murine Pex3p with the human Pex3p sequence. These two proteins revealed an overall amino acid identity of 93.8%. The degree of identity to the Pex3 proteins of other species is 22.3% to *Pichia pastoris* (455 amino acids), 20.9% to *Hansenula polymorpha* (457 amino acids), 19.9% to *Saccharomyces cerevisiae* (441 amino acids), and 18.6% to *Kluyveromyces lactis* (483 amino acids). Protein similarity scores were determined using the Align program from EERIE (Nimes, France). A comparative analysis of Pex3 proteins using the TMpred server of the Bioinformatics group at ISREC (Epalinges, Switzerland) predicted two hydrophobic transmembrane regions that are located in the N-terminal half of the protein in all species.

To determine the exon-intron structure of the mouse *PEX3* gene, a 129/Ola mouse genomic DNA cosmid library was screened with a mouse cDNA probe. One positive clone, mcos3, covering about 17 kb of the *PEX3* gene including exon 3 to exon 12 was isolated. To amplify the introns, multiple exon-exon PCR reactions were performed using mcos3 cosmid DNA, mouse genomic DNA from 129/Ola mouse embryonic stem cells or both as a template. Exons 1 and 2, which were not present on the clone mcos3, were exclusively amplified from mouse genomic DNA. Overlapping fragments spanning the complete cod-

ing region were generated and sequenced from either end using the amplification primers in order to identify the intron-exon boundaries. Comparison of the obtained genomic sequence with the cDNA sequence revealed that the mouse *PEX3* gene consists of 12 exons and 11 introns (Figure 1). The exon sizes range from 44 bp (exon 4) to 166 bp (exon 8). All exon-intron junctions conform to the GT-AG rule (Figure 2). Five introns (intron 4, 5, 6, 7, and 9) were fully sequenced and the exon-flanking intronic sequences of all other introns were determined. The approximate sizes of introns not fully sequenced were estimated by agarose gel electrophoresis of the corresponding PCR products. The total size of the mouse *PEX3* gene is approximately 30 kb (Figure 1).

To characterize the putative promoter region, 2 kb of genomic sequence immediately upstream of the transcriptional start sequence were obtained by long template PCR. Analysis of this 5' flanking sequence for consensus promoter elements was performed using the Transcription Factor Database (TFD) (Ghosh *et al.*, 1991) and the Signal Scan server of BIMAS (Prestridge *et al.*, 1991). Canonical TATA and CCAAT boxes, as a characteristic feature of housekeeping genes which are expressed at low levels in essentially all tissues, were not found. The region close to the transcription initiation site was shown to be relatively GC-rich, exhibiting a GC content of about 60% from –250 to –1. This sequence contains a putative binding site for the transcription factor Sp1 at –30 to –25, which was found with identical sequence and similar location in the orthologous human gene (Muntau, unpublished). At approximately 1.8 kb upstream of the transcription start site we detected a polymorphic CT repeat. By analyzing several subclones, repeat lengths between 17 and 27 – with the exception of 21 and 22 repeats – were identified.

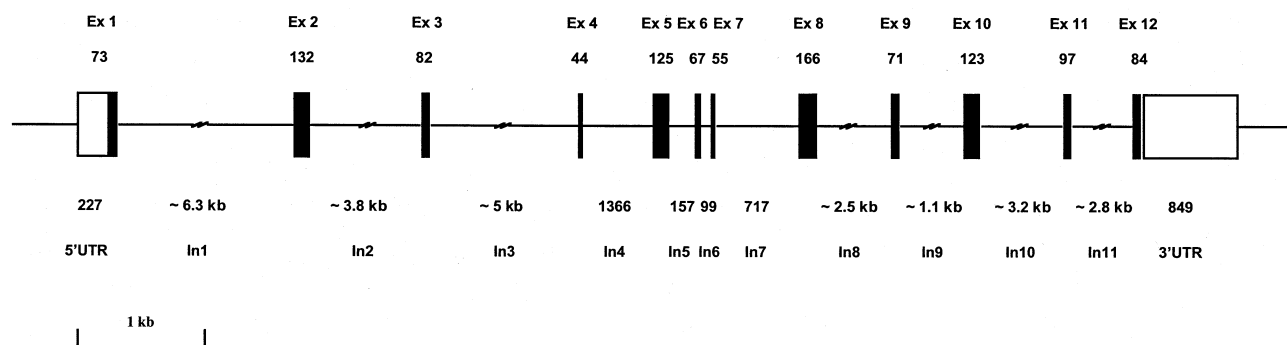


Fig. 1 Genomic Organization of the Mouse *PEX3* Gene.

The gene consists of twelve exons (closed boxes) and eleven introns (solid lines between the exons). Exon sizes in base pairs are indicated above, intron sizes below the horizontal line. The 5' and 3' UTRs are shown as open boxes.

Methods: mouse cosmid DNA was prepared using ion exchange column purification (Midiprep Kit, Qiagen). Multiple exon-exon PCR reactions were performed using mcos3 cosmid DNA, mouse genomic DNA from 129/Ola mouse embryonic stem cells or both as a template. The resulting PCR products were sequenced from either end using the amplification primers. The sequence containing the putative promoter region was obtained by long template PCR (Expand Long Template PCR System, Boehringer Mannheim) using adaptor-ligated genomic DNA fragments (Mouse Genome Walker Kit, Clontech) according to the manufacturer's procedure. Two successive PCR reactions were performed using the anchor-specific primer AP1 and a mouse *PEX3*-specific reverse primer (m128, 5'-ACT GTG CCC AGG AAG ATG C-3'), and then AP2 combined with a nested reverse primer from the coding region (m81, 5'-CCA CAT TGA TCT CAG CAT CTC C-3'). The PCR products obtained were purified from the gel (QIAquick Gel Extraction Kit, Qiagen), directly sequenced by fluorescent dye terminator technology and separated on an ABI 377 sequencer. EMBL/GenBank sequence accession numbers: AF152996 and AF162890 through AF162896.

		Intron			
		5' splice site (donor)		3' splice site (acceptor)	
Mm	CTCGGAG	GTGAGTTGACAACA	---In1---	TTTAATGATTTTATAG	GAGTATA
Hs	CTTGGAG	GTGGGTGACAACGT	---In1---	TTTAATGATTGTAG	GAGTATA
Mm	ATGACAG	GTAAGCATGCGCGC	---In2---	CTGTTGAAAAGCAG	TGCTGTC
Hs	ATGACAG	GTAAGACAGGGAGA	---In2---	TGTTTGTATTACAG	TGCTGTC
Mm	AAAGCAG	GTGAAAGCGCACTG	---In3---	ATTTTTTTCTTTAG	GCCTTCA
Hs	AAAACAG	GTAAATGCAAGTTA	---In3---	ATTTTGTCTTTAG	GCCTTCA
Mm	ATAATAA	GTAAGTCCAAATAT	---In4---	TCTTCTCTCTGAAG	GTTTCAC
Hs	ATAATAA	GTAAGCCTGCATAT	---In4---	TTTCTCTCTGTGAAG	GTTTCAC
Mm	TGGCACT	GTAAGTTTAACAGA	---In5---	TTCTGTTTGCACAG	ACCGTTC
Hs	TGGCACT	GTAAGTTTAATAGA	---In5---	TCTGTTTTATACAG	ACAATTC
Mm	GGAGACG	GTAAGATTCCAGTC	---In6---	TTTTTGTTTGATAG	GCCTCAC
Hs	GGAGATG	GTAAGATTCTTATT	---In6---	TTTTTCTTTAATAG	GCCTGAC
Mm	TAGGAAG	GTATGCTATGTCCT	---In7---	TCCTCTATTTCCAG	TGTCTCT
Hs	TAGGAAG	GTAAGGCATTTTTTC	---In7---	TTCTCTGTTTCTAG	TGTTTCT
Mm	AGCTCAG	GTAAGTAAATTCATA	---In8---	TTTTGATTTTACAG	GCCTATG
Hs	AGCTCAG	GTAAGTAAATTCATA	---In8---	TTTTAATTTCTATAG	GCCTGTG
Mm	TGGAAAG	GTATGTAGAGTGCA	---In9---	CTTTTTTTATCTAG	TCCAGAT
Hs	TGGAAAG	GTATGTATACTTCA	---In9---	CTTACTTTATATAG	CCCAGAT
Mm	TAAACAG	GTAAGTTGGAATGT	---In10---	TAATTTGTTTTCAG	TCTGTCC
Hs	TGAATAG	GTAAGATGACATAT	---In10---	TAATGATTTTTCAG	TCTTTCC
Mm	TGTGCAG	GTAAGTGAGAAGCT	---In11---	ATGGTTGTTACCAG	GATCTGC
Hs	TGTTTCA	GTAAGAAGAAAGCT	---In11---	ATCATCTTTGCTAG	GATCTGT
Cs		GTAAGT G		TTTTTTTTTTTNCAG CCCCCCCCC	

Fig. 2 Comparison of the Exon-Intron Boundaries of the Mouse and Human *PEX3* Gene.

The consensus sequences (CS) of the 5' and 3' intron splice sites are given below, where N is any nucleotide and, where two nucleotides are given, the one above is more common (Shapiro and Senapathy, 1987).

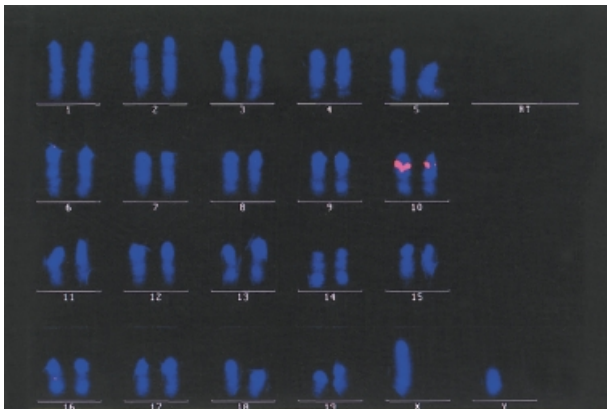


Fig. 3 Chromosomal Localization of the Mouse *PEX3* Gene. Specific hybridization signals were identified on band A of chromosome 10 using fluorescence *in situ* hybridization of metaphase chromosomes.

Methods: metaphase chromosome preparations were obtained from mouse bone marrow cells according to standard procedures. The cosmid clone mcos3, containing about 17 kb of the murine *PEX3* gene, was labeled with biotin-14-dUTP (Life Technologies) by nick-translation and was detected with streptavidin-Cy3 (Amersham Life Science). Metaphase chromosomes were analyzed under an Axiopog microscope (Zeiss) and pictures were taken by digitizing the microscopic image with the computer program ISIS3 (Metasystems).

A visual inspection of the promoter region for possible peroxisome proliferator-responsive elements (PPRE) or oleate-responsive elements (ORE) did not reveal motifs that show a strong similarity to the consensus sequences (Zhang *et al.*, 1992; Karpichev *et al.*, 1997).

The chromosomal localization of the *PEX3* gene was determined by fluorescence *in situ* hybridization (FISH) analysis. The cosmid clone mcos3, containing about 17 kb of the mouse *PEX3* gene, was labeled with biotin. By hybridization of this probe to mouse metaphase chromosomes, *PEX3* was localized to band A on chromosome 10 (Figure 3). This region has syntentic relationship with the human chromosome region 6q23-6q24 where the human *PEX3* gene has been located by FISH analysis (Muntau, unpublished) and by large-scale genome sequencing (NCBI, GenBank accession number AL031320).

PEX3 mRNA expression in various mouse tissues was examined by Northern blot analysis using a 1.6 kb mouse cDNA as a probe. A human actin probe was used as control for the loaded amount of RNA. The presence of *PEX3*-specific mRNA in all tissues examined, with the strongest expression in liver and testis, was demonstrated. In heart, brain, spleen, lung, liver, skeletal muscle, and kidney, signals at 1.6 and 2.3 kb were detected, with the 2.3 kb signal being predominant in these tissues (Figure 4). These frag-

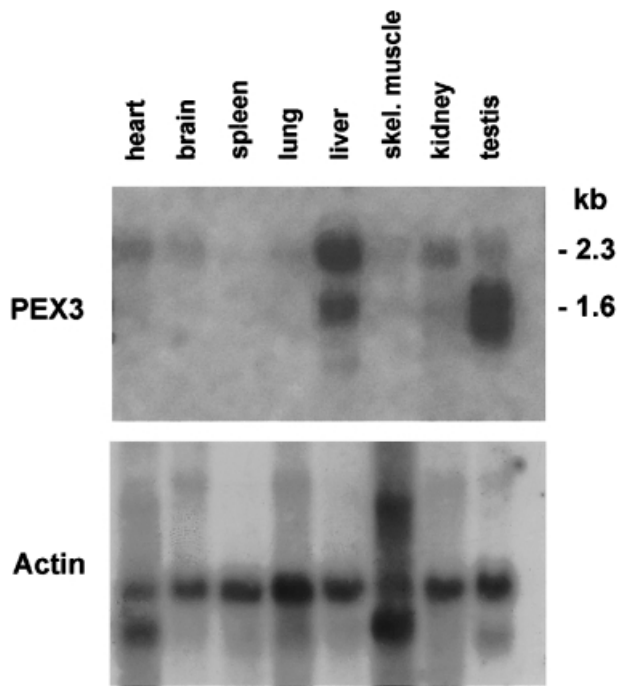


Fig. 4 Expression of the *PEX3* Gene in Various Mouse and Rat Tissues.

A Northern blot of mRNA from several murine tissues was hybridized with the 1.6 kb mouse cDNA probe. A human actin probe was used as control for the loaded amount of RNA. Fragment sizes are indicated on the right.

Methods: *PEX3* mRNA expression in various mouse tissues was examined using a BALB/c mouse multiple tissue Northern blot containing 2 μ g poly(A)⁺ mRNA per lane (Clontech). A 1.6 kb cDNA fragment was isolated with *NotI* and *XhoI* from the mouse EST clone m1 and radiolabeled with [α -³²P]dATP by random priming. The blot was hybridized as described elsewhere (Braun *et al.*, 1994) and exposed to an X-ray film for 10 days. Signals obtained were compared to those detected by a human actin cDNA probe.

ments seem to be derived from alternative polyadenylation at +1341/1376 and +2017 bp described above. In testis, in addition to the 2.3 kb transcript several shorter fragments were observed with lengths between 1.4 and 1.7 kb. This was the only tissue in which the shorter transcripts were expressed at a higher intensity. It might be interesting to note that a marked morphological heterogeneity of peroxisomes has been described in cultured mouse testicular Leydig cells (Litwin and Bilinska, 1995). *PEX3* might potentially be involved in the formation of small local networks of interconnected tubular peroxisomes observed in this study.

Pharmacological modification of the expression levels of specific peroxins might be a valuable addition to treatment strategies for human peroxisomal biogenesis disorders. Fenofibrate, which was proven to be a potent peroxisomal proliferator in rodents, is a broad-spectrum lipid-lowering agent widely used in patients with coronary heart disease (Packard *et al.*, 1998). To compare *PEX3* mRNA expression in rats before and after fenofibrate treatment, Northern blot analysis of rat tissues using a human 2.1 kb *PEX3* probe was performed. Signals obtained were com-

pared to those detected with a rat glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) control probe. As a positive control for the effectiveness of the fenofibrate treatment, hybridization with a mouse acyl-CoA oxidase (*AOX*) probe was performed. As in the mouse, *PEX3*-specific mRNA in rat was also expressed in all tissues examined. The amount of lower transcript was slightly increased in liver but overall the transcripts showed a more even distribution than in mouse (Figure 5). The examination of rat testis tissue has still to be performed. The fenofibrate-treatment did not affect the level of *PEX3* mRNA expression, indicating that transcription of *PEX3* is not induced by fenofibrate in rats (Figure 5). In contrast, the level of peroxisomal acyl-CoA oxidase (*AOX*) mRNA used as a marker of peroxisomal proliferation was strongly enhanced in liver, kidney, intestine and heart, proving the effectiveness of fenofibrate treatment (Figure 5). Fenofibrate is known to strongly increase the level of peroxisome proliferator activated receptors in rats (Gebel *et al.*, 1992). Interestingly, the mouse promoter does not exhibit can-

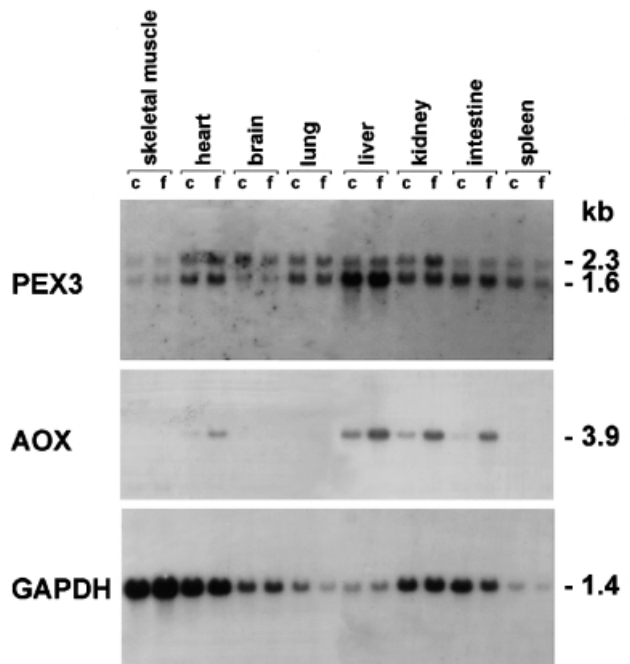


Fig. 5 Northern Blot Analysis in Various Tissues from Fenofibrate-Treated and Untreated Rats.

Hybridization was performed using the 2.1 kb human *PEX3* cDNA probe. Signals obtained were compared to those detected with a rat glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA control probe. The level of peroxisomal acyl-CoA oxidase (*AOX*) mRNA was used as marker for peroxisomal proliferation. Fragment sizes are indicated on the right. c: untreated, f: fenofibrate-treated rats.

Methods: Northern blot analysis of rat tissues using a human 2.1 kb *PEX3* probe was performed as previously described (Albet *et al.*, 1997). Signals obtained were compared to those detected with a rat glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) control probe. As a positive control for the effectiveness of the fenofibrate treatment, hybridization with a mouse acyl-CoA oxidase (*AOX*) probe prepared from a 246 bp coding fragment was performed as described elsewhere (Albet *et al.*, 1997).

onical peroxisome proliferator-responsive elements. Assuming that this is also the case in rat, this might explain the lack of induction of rat *PEX3* by fenofibrate. It has been reported previously that fenofibrate strongly induced the expression of two other peroxisomal membrane proteins of the ABC-transporter superfamily, PMP70p and ALDRp, in rat and mouse (Albet *et al.*, 1997; Holzinger *et al.*, 1998; Berger *et al.*, 1999). These data are in agreement with the hypothesis that proteins which are directly or indirectly involved in peroxisomal metabolism are pharmacologically inducible, whereas proteins involved in the peroxisomal biogenesis, such as Pex3p, can not be induced. Confirmation of these findings would have important consequences for the development of new treatment strategies.

Based on the findings in yeast, *PEX3* is a candidate gene for human peroxisomal biogenesis disorders. *PEX3* mutations have not been identified so far. Among the considerable number of *PEX* genes, mouse models have only been described for *PEX2*, which encodes a zinc-binding integral membrane protein (Faust and Hatten, 1997), and *PEX5*, which encodes the import receptor for most peroxisomal matrix proteins (Baes *et al.*, 1997). However, a mouse model with targeted disruption of one of the *PEX* genes involved in the initial steps of peroxisomal biogenesis is not yet available. The data reported here might contribute to further elucidation of the basic aspects of formation and function of peroxisomes, and help to develop treatment strategies for peroxisomal biogenesis disorders, which are still leading to early death in affected children.

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