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Genomic organization, chromosomal localization and tissue specific expression of the murine *Pxmp2* gene encoding the 22 kDa peroxisomal membrane protein (Pmp22)

Georg H. Lüers[a,*], David M. Otte[a], Suresh Subramani[b], Thomas Franz[a]

[a]*University of Bonn, Institute for Anatomy, Nussallee 10, D-53115 Bonn, Germany*
[b]*Department of Biology, University of California at San Diego, La Jolla, CA 92093-0322, USA*

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Abstract

Peroxisomes are subcellular organelles with important functions in lipid metabolism that are found in virtually all eucaryotic cells. The peroxisomal membrane contains a number of integral and peripheral membrane proteins involved in the import of peroxisomal matrix proteins and the transport of metabolites across the membrane. The most abundant peroxisomal membrane protein (Pmp) in rat peroxisomes is Pmp22, a 22 kDa protein of unknown function that is encoded by the *Pxmp2* gene. To investigate the function of the *Pxmp2* gene, we have initiated mouse knockout studies. The expression level of the *Pxmp2* mRNA in mice was investigated by Northern blot analysis. *Pxmp2* RNA was shown to be differentially expressed with highest expression levels in liver, kidney and in heart tissue. Comparison with other peroxisomal marker genes revealed that the expression of *Pxmp2*, *Pmp70* (*Pxmp1*) and catalase was regulated independently. Using 5′ and 3′ RACE we have cloned the full-length cDNA of murine *Pxmp2* which comprises 863 nucleotides and have isolated a genomic clone containing the entire murine *Pxmp2*. We have analyzed the complete intron/exon structure of the *Pxmp2* gene which contains five exons spanning about 11 kb on the genomic clone. All intron/exon splice junctions conform to the GT/AG rule. Sequence analysis of the *Pxmp2* 5′ flanking region revealed that it was devoid of a TATA box, but characteristic promoter elements were identified within 250 base pairs upstream of the transcriptional start site. Using a mouse/hamster radiation hybrid panel, *Pxmp2* was localized on mouse chromosome 5 at 59 cM.

Keywords: Peroxisome; Intron/exon structure; Radiation hybrid mapping; *Pxmp2*

1. Introduction

Peroxisomes are organelles present in virtually all eucaryotic cells. Their importance is highlighted by a number of genetically determined human disorders with impaired peroxisomal functions (Moser, 2000; Fujiki, 2000), such as Zellweger Syndrome and neonatal adrenoleukodystrophy among others. Peroxisomes contain enzymes for a variety of functions including important pathways in lipid and purine metabolism and for the production and degradation of hydrogen peroxide (van den Bosch et al., 1992). They are surrounded by a single membrane with a unique composition of peripheral and integral membrane proteins (Fujiki et al., 1982). Several minor peroxisomal membrane proteins were found to be mutated in peroxisomal disorders providing clues for their function (Fujiki, 2000). The function of the Pmp22, one of the most abundant peroxisomal membrane proteins, however, remains unknown.

Pmp22 is an integral Pmp of 22 kDa (Hashimoto et al., 1986; Hartl and Just, 1987). It is encoded by the *Pxmp2* gene, translated on free polysomes without further processing (Suzuki et al., 1987) and is translocated into the peroxisomal membrane posttranslationally (Fujiki et al., 1989; Diestelkötter and Just, 1993). The cDNAs of the rat and the murine *Pxmp2* encode a protein of 194 amino acids (Kaldi et al., 1993; Bryant and Wilson, 1995) with four putative transmembrane domains. *Pxmp2* RNA has previously been shown to be differentially expressed with high levels of expression in liver and kidney (Bryant and Wilson, 1995). Compared with other peroxisomal proteins, the expression of Pmp22 in rat liver is only moderately induced upon treatment with the peroxisome proliferator

Abbreviations: BAC, bacterial artificial chromosome; DIG, digoxigenin; ORF, open reading frame; PCR, polymerase chain reaction; Pmp, peroxisomal membrane protein; RACE, rapid amplification of cDNA ends; UTR, untranslated region

* Corresponding author. Tel.: +49-228-739008; fax: +49-228-733346.
E-mail address: luers@uni-bonn.de (G.H. Lüers).

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di(2-ethylhexyl)phthalate (DEHP) (Suzuki et al., 1987; Hartl and Just, 1987). Under conditions of cell proliferation due to liver regeneration, expression of Pmp22 is reduced in contrast to the expression of other peroxisomal membrane proteins such as Pmp26 or Pmp70 (Lüers et al., 1990). Van Veldhoven et al. (1987) have speculated on a pore forming activity for this protein because a protein fraction containing Pmp22 and other Pmps was shown to confer leakiness for small molecules such as sucrose to liposomes. In order to further analyze the function of the *Pxmp2*, we have initiated knockout studies in mice.

In this study we present the cloning of the full-length cDNA of the murine *Pxmp2*. We have also isolated a genomic fragment containing the entire murine *Pxmp2* and have analyzed the complete intron/exon structure as well as its chromosomal localization. The expression pattern of the *Pxmp2* was analyzed in Balb/c mice.

2. Materials and methods

2.1. Materials and general procedures

Escherichia coli strain XL1blue (Stratagene, Heidelberg, Germany) was used in all cloning procedures involving plasmid propagation. PCR, restriction analysis, agarose gel electrophoresis, Southern blotting, cloning techniques and transformation of *E. coli* were performed according to standard protocols (Sambrook et al., 1989). Genomic DNA was isolated using the DNeasy kit from Qiagen, (Qiagen, Hilden, Germany). The KB-500 DNA isolation kit from Genome Systems (St. Louis, MO, USA) was used for purification of BAC plasmid DNA and the Plasmid midi kit (Qiagen, Hilden, Germany) was used for isolation of DNA of all other plasmids. DNA fragments were extracted from agarose gels using the QIAEXII kit (Qiagen, Hilden, Germany). Total RNA was prepared from cells using RNeasy mini Kit or from tissues using RNeasy midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

Primers used in this study for cloning of cDNA or synthesis of specific probes were:

GLO17 ATGGCACCTGCGGCGTCCAGGC;
GLO18 TCCCCAGAGACGCCAGGTAGGC;
GLO19 CGGCTTCTAGAAGTCAGTGGGC;
GLO23 TTGACGGCACGGAACACCTCGC;
GLO26 TCGTAGAGGTACCGTGTGAGCC;
GLO111GTAGTGACTCAGTGGACCTGTG;
GLO122CTTGGCAACAAAGACACTGACG;
GLO148ATGTATGTGCGTGGGTATAGG;
GLO149AGTAGCGTATATGCCTCCTGG;
GLO168TTGCTAGCGATGGCACCTGCGGCGTCC;
GLO171CAGAATTCTCACTTCCCCAGAGACGCCAGG.

All PCR primers were obtained from MWG Biotech (Ebersberg, Germany). Sequencing of DNA was performed at SeqLab (Göttingen, Germany) using dye-terminator-technology. Sequence analysis was performed using the OMIGA 2.0 software (Oxford molecular, Oxford, England). Restriction enzymes and DNA modifying enzymes were obtained from MBI Fermantas (St. Leon-Rot, Germany) unless indicated otherwise. All other chemicals were obtained from Sigma (Munich, Germany) unless indicated otherwise.

2.2. cDNA cloning

The full-length *Pxmp2* cDNA was cloned by 5′ and 3′ RACE using the SMART RACE cDNA Amplification Kit from Clontech (Heidelberg, Germany) and superscript II from Gibco (Eggenstein, Germany) as the reverse transcriptase. RNA was prepared from a culture of primary embryonal (E15) murine strain 129/Sv fibroblasts and from adult strain 129/Sv liver. The 5′RACE was performed with both RNA preparations as template using the gene specific primers GLO122 for the RACE and GLO111 for the subsequent nested PCR. The 3′RACE was performed with the 129/Sv fibroblast RNA as the template using primer GLO17 for the RACE, and GLO19 for the nested PCR. The RACE reactions were performed as recommended by the manufacturer using a touch down PCR protocol covering the temperature range from 58 to 52°C. The nested PCR was performed according to the same protocol using Pfu DNA polymerase (Stratagene, Heidelberg, Germany). PCR products, obtained from the RACE reactions, were isolated from agarose gels and ligated into *Eco*RV-linearized pBluescript II SK (Stratagene, Heidelberg, Germany). Ten clones were sequenced to identify the 5′ and 3′ UTR. The cDNA preparation obtained from adult strain 129/Sv liver RNA was used as template for amplification of the *Pxmp2* open reading frame (ORF) with primers GLO168 and GLO171 using Pfu DNA polymerase. The expected PCR product of 600 bp was isolated and ligated into *Eco*RV-linearized pBluescript II SK for sequencing.

2.3. Cloning of the genomic Pxmp2 and structure analysis

Primers derived from the published partial cDNA sequence (Bryant and Wilson, 1995) were used to amplify intron 4 with flanking exon sequences from genomic murine 3T3 DNA as the template. A 600 bp fragment (indicated in Fig. 1) containing a part of intron 4 with flanking exon 4 sequence was used to screen a BAC library of genomic mouse strain 129/Sv DNA. The hybridization screen was performed at Genome Systems (St. Louis, MO, USA). Four distinct overlapping BAC clones with insert sizes of 100 to 120 kb, all containing the entire *Pxmp2* gene, were obtained. One clone was analyzed by Southern blot using a cDNA fragment (bp 1–192 of the ORF) and the genomic DNA fragment employed for screening of the BAC library as probes in order to generate a crude restriction map of the locus. Several fragments of genomic DNA, a 5.3 kb *Apa*I- (pGHL80), a 7 kb *Eco*RI- (pGHL155), two 4.5 kb *Eco*RI/

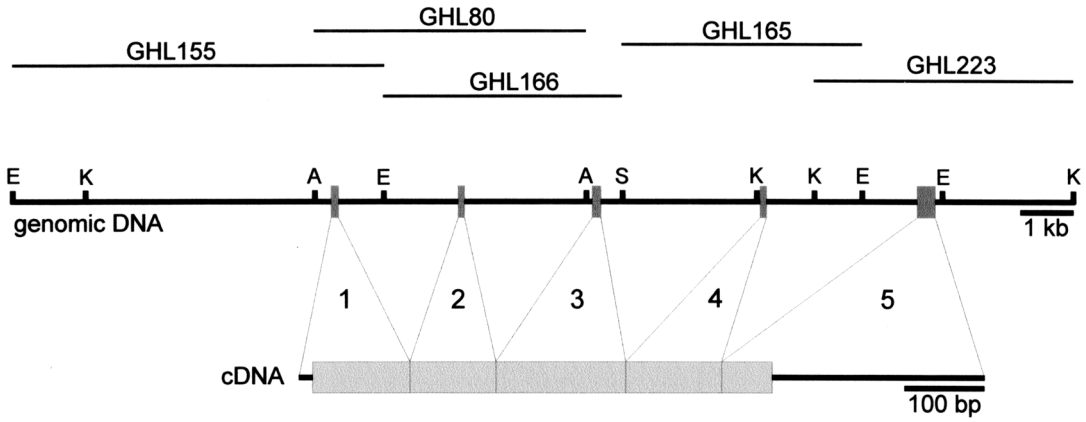

Fig. 1. Genomic organization of the murine *Pxmp2* gene. The thin bars on top indicate the localization of genomic fragments that were subcloned for detailed sequence and restriction analysis. On the line representing the genomic DNA, exons are shown as filled boxes. Restriction sites are: A, *Apa*I; E, *Eco*RI; K, *Kpn*I; and S, *Sal*I. For the cDNA, the ORF is represented by the filled box whereas the untranslated regions are indicated by the thick line. Exons (1–5) are indicated in Arabic numbers.

*Sal*I- (pGHL165 and pGHL166) and a 5 kb *Kpn*I-fragment (pGHL223) were cloned into pBluescript II SK (Stratagene, Heidelberg, Germany). These plasmids were used for sequence analysis and detailed restriction enzyme mapping. Some restriction sites as well as the localization of the genomic subclones are indicated in Fig. 1. The genomic BAC clone was used for intron-spanning PCR with various primers derived from the cDNA sequence to obtain complete introns and their sizes.

2.4. Radiation hybrid-mapping of the chromosomal localization of the Pxmp2

The chromosomal localization of the *Pxmp2* was analyzed using a T31 mouse/hamster radiation hybrid panel consisting of 100 different hybrid DNA samples from Research Genetics (Huntsville, Canada). Primers GLO148 and GLO149 were used to specifically amplify a 600 bp fragment from intron 4 of the murine *Pxmp2* by PCR. Cycling conditions were 30 s at 94°C, 30 s at 57°C and 2 min at 72°C for 30 cycles using Taq DNA polymerase with recommended reaction buffers. The retention rate of genomic *Pxmp2* sequences was 33% within the radiation hybrid panel. Analysis of the chromosomal localization by comparison of our *Pxmp2* retention pattern with the pattern of known genomic markers was performed at the Jackson Laboratory at http://www.jax.org/resources/. The locus of the *Pxmp2* was submitted to the mouse genome database (MGD) at http://www.informatics.jax.org/mgihome/nomen/ .

2.5. Northern blot analysis

Total RNA was separated on 1.2% agarose gels in the presence of formaldehyde and was blotted onto Qiabrane filters (Qiagen, Hilden, Germany) by vacuum blotting for 90 min using a vacuum blotter (Model 784) from Bio-Rad (Munich, Germany). For analysis with cDNA probes, the filters were prehybridized at 50°C for 1 h in 50% (v/v) formamide, 5× SSC, 50 mM sodium phosphate, pH 7, 2% (w/v) blocking solution (Roche, Mannheim, Germany), 1% (w/v) N-lauroylsarcosine and 7% (w/v) SDS. After addition of 20 ng/ml of specific digoxigenin (DIG)-labeled probes, the filters were hybridized at 50°C overnight. For analysis with a cRNA probe, the filters were incubated at 68°C and the buffers were lacking sodium phosphate and contained only 0.02%SDS (w/v) for prehybridization supplemented with 100 ng/ml of probe for hybridization. The hybridized probes were detected by binding of anti-digoxigenin antibodies conjugated with alkaline phosphatase as described by the manufacturer (Roche, Mannheim, Germany). Alkaline phosphatase activity was visualized by light emission after incubation of the filter with CDP-star (Roche, Mannheim, Germany). Filters were exposed to Kodak X-OMAT AR films for 5 up to 30 min.

Specific probes for the *Pxmp2* and PMP70 genes were obtained by PCR in the presence of DIG-dUTP (Roche, Mannheim, Germany). PCR was performed with primers GLO17 and GLO18 using plasmid GHL13, which contains the entire ORF of the murine *Pxmp2* gene, as template and with primers GLO23 and GLO26 using plasmid GHL39, which contains 470 bp of the 5′end of the ORF of the murine *Pmp70* gene, as template. For detection of the catalase mRNA, we used a digoxigenin-labeled antisense cRNA probe specific for rat catalase that was prepared as described by Zhan et al. (1997). Digital images of the 18S rRNA bands were analyzed densitometrically using the Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

3. Results

3.1. Cloning of the full-length Pxmp2 cDNA

We cloned the 863 bp full-length *Pxmp2* cDNA of the

Table 1
Intron/exon structure of the murine *Pxmp2*

Exon/intron	Exon (bp)	Intron (kb)	Nucleotide position in cDNA (bp)[a]	Splice acceptor	Exon boundary	Splice donor
1	139	2.2	−17–122		..GCAG	gtgggc
2	108	2.4	123–230	aaacag	CGGC..ATGG	gtaagt
3	163	3.1	231–393	gtccag	GTTG..GGAG	gtaagt
4	120	2.8	394–513	ctgcag	GGGA..GCAG	gtgaga
5	333[b]		514–846	tggcag	TTCC..	

[a] The A of the initiation codon (ATG) is designed +1.
[b] Length to the last nucleotide preceding the poly(A) sequence.

murine strain 129/Sv using 5′ and 3′ RACE as described in Section 2. The 579 bp open reading frame (ORF) encodes a protein of 193 amino acids with a predicted molecular weight of 22.1 kDa. Comparison of this sequence with the published sequence of the ORF of Swiss Webster mice (Bryant and Wilson, 1995) revealed differences in 11 nucleotide positions causing two silent mutations, four amino acid exchanges (Q59R, R60K, R64Q and L65N) and the deletion of a K at position 58.

To identify the 5′ UTR of the *Pxmp2*-RNA, seven separate clones representing distinct products from 5′RACE reactions, three derived from 129/Sv embryonal fibroblast RNA and four derived from 129/Sv adult liver RNA, were sequenced. Sequences varied only in one nucleotide following the smart II cDNA primer at position -18 from the putative translational start predicting a length of the 5′ UTR of 17 nucleotides. To determine the length of the 3′ UTR, three distinct clones derived from 3′RACE using 129/Sv fibroblast RNA were analyzed. Sequences varied only within the five nucleotides preceding the poly A sequence thus predicting a length of the 3′UTR of 267 nucleotides. Sequence analysis of the 3′UTR revealed a poly-adenylation signal in the range of 14 to 19 nucleotides upstream of the poly A sequence. The sequence of the murine 129/Sv *Pxmp2* cDNA has been submitted to GenBank accession number AF309644.

3.2. Cloning and characterization of the genomic Pxmp2 sequence

BAC clones containing the genomic murine strain 129/Sv *Pxmp2* were obtained and fragments of 5–7 kb, covering the complete gene, were subcloned as described in Section 2 (Fig. 1). The genomic organization was determined by intron-spanning PCR using primers specific to cDNA sequences with the genomic BAC clone as template and sequence comparison of the cDNA with the corresponding genomic DNA. The genomic subclones were used to sequence all exons and the flanking intron regions. The *Pxmp2* was found to consist of five exons ranging from 108 to 333 bp and four introns ranging from 2.2 to 3.1 kb. Sequences of the cDNA were spread over 11 kb of genomic DNA (Fig. 1). Sequence analysis of intron/exon boundaries and sizes of introns and exons are summarized in Table 1. All splice donor/splice acceptor sequences conformed to the GTAG rule for intron boundaries.

3.3. Chromosomal localization of the Pxmp2

We screened a mouse/hamster radiation hybrid panel for the presence of genomic sequences of the murine *Pxmp2* gene. By comparison with the distribution of known chromosomal markers within the radiation hybrid panel, the *Pxmp2* gene could be localized to mouse chromosome 5. The highest anchor LOD's were 13.8 for marker D5Mit314 and 13.4 for the marker D5Mit315. The resulting best-fit localization is between D5Mit 21 cR proximal and D5Mit314 19cR distal in the central region of chromosome 5 at about 59 cM. The chromosomal locus of the *Pxmp2* was submitted to the mouse genome database (MGD at: nomen@informatics.jax.org).

3.4. Analysis of the putative promoter region

About 2.1 kb of the 129/Sv genomic DNA upstream of the transcriptional start of the *Pxmp2* were sequenced and analyzed for the presence of consensus sequences for transcription factors binding sites and basic promoter elements. Within the region of 250 bp upstream of the transcriptional start, a cluster of GC-boxes for Sp1 binding as well as CAAT motifs, both components of the basic transcription initiation complex, were identified (Fig. 2). A TATA box was absent in this region. The 2.1 kb upstream of the transcriptional start did not contain a typical peroxisome proliferator response element (PPRE).

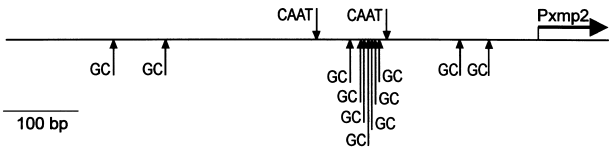

Fig. 2. Analysis of the *Pxmp2* upstream region. A part of the genomic *Pxmp2* upstream sequence is represented by the line. The horizontal arrow indicates the transcriptional start. Putative binding sites for transcription factors are marked by vertical arrows. Note the cluster of GC-boxes about 200–250 bp upstream of the transcriptional start.

3.5. Expression pattern

The expression of peroxisomal marker genes was analyzed by Northern blotting of total RNA from various Balb/c tissues. Loading of comparable amounts of total RNA was verified by densitometric analysis of the 18S rRNA bands. The integrated optical density of the rRNA bands varied within 15% compared with the 18S rRNA band of lung tissue (Fig. 3, bottom panel, lane 1). The *Pxmp2* RNA was differentially expressed with highest expression levels in liver, kidney and heart tissue (Fig. 3). Lesser amounts of *Pxmp2* mRNA were detected in lung tissue but with the digoxigenin-labelled probe used, expression of *Pxmp2* was below detection limits in spleen, forebrain and cerebellum. Furthermore, we estimated the length of the *Pxmp2* mRNA to be within the range of 800 to 850 bp using commercially available molecular weight markers (Roche, Mannheim, Germany) on Northern blots. The same RNA's were analyzed for expression of *Pmp70* (*Pxmp1*), another major peroxisomal membrane protein, and for catalase, a marker enzyme for the peroxisomal matrix compartment. Both *Pmp70* and catalase were also found to be highly expressed in liver and kidney. Compared to *Pxmp2*, however, the expression of *Pmp70* and catalase in heart tissue was only moderate (Fig. 3), indicating an increased relative expression level of *Pxmp2* in heart tissue.

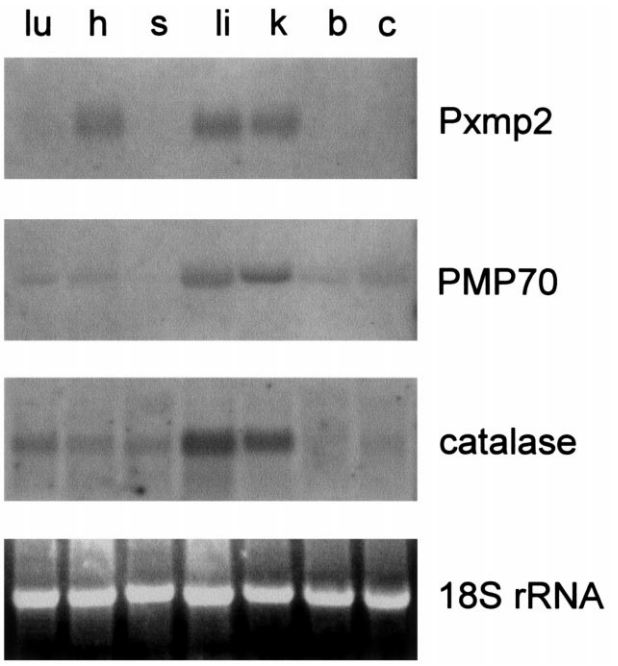

Fig. 3. Northern blot analysis of peroxisomal marker genes in various mouse tissues. The bottom panel shows the 18S rRNA bands of the ethidium bromide-stained gel to indicate equal loading of the gels with total RNA. All other panels show Northern blots hybridized with specific probes for genes indicated. The mouse (Balb/c) tissues are: lu, lung; h, heart; s, spleen; li, liver; k, kidney; b, forebrain; and c, cerebellum.

4. Discussion

We cloned and sequenced the full-length cDNA of the *Pxmp2* of the 129/Sv mouse genome. The 5′ UTR was shown to contain 17 nucleotides. In order to verify the length of the 5′UTR, we analyzed seven independent clones all confirming the same sequence for the 5′UTR. These data corroborate, that our cDNA (863 bp) indeed represents the full-length of the transcribed message. Splice variants were not detected in the tissues examined.

A number of nucleotide differences were found upon comparison of our 129/Sv cDNA with the published sequence of the ORF from Swiss Webster mice (Bryant and Wilson, 1995). We sequenced all exons of the *Pxmp2* gene using strain 129/Sv genomic DNA as template and found, that the exon sequences were precisely represented in our cDNA sequence. The relevance of the cDNA sequence differences among 129/Sv and Swiss Webster mice cannot be assessed at this time and may reflect genetic heterogeneity between both strains.

We also investigated the expression pattern of the *Pxmp2* in organs obtained from adult inbred Balb/c mice. The highest levels of *Pxmp2* expression were found in liver and kidney, which is in agreement with data presented by Bryant and Wilson (1995) for Swiss Webster mice. Using RTPCR these authors also show fairly high expression of *Pxmp2* in nervous tissue such as cerebral cortex, cerebellum or spinal cord and a very low expression level in heart tissue. In contrast, using Northern blot analysis, we demonstrate that in Balb/c mice *Pxmp2* is also highly expressed in heart tissue but is below detection limits in the forebrain or the cerebellum. These distinct expression patterns either reflect heterogeneity between different mouse strains or they are due to differential gene regulation and therefore reflect different functional states.

We have identified a putative promoter region within 2.1 kb upstream of the transcriptional start by sequence analysis which revealed several clusters of transcription factor binding sites within this region. Interestingly, we could not find a TATA box within this genomic sequence. Promoter regions lacking TATA boxes have previously been described for housekeeping genes (Azizkhan et al., 1993; Haun et al., 1993), which may indicate that *Pxmp2* shares features of control of expression with those genes.

Analysis of the putative promoter region did not show any consensus binding site (Green, 1995; Michalik and Wahli, 1999) for peroxisome proliferator activated receptors, transcription factors known to be involved in regulation of expression of some peroxisomal genes. For the peroxisome proliferator responsive elements, however, it was shown that consensus sequences can be localized further upstream from the transcriptional start than we have so far analyzed (Zhang et al., 1992).

Comparison of expression patterns of *Pxmp2*, *Pmp70* (*Pxmp1*) and catalase revealed that these peroxisomal genes were regulated differentially (Fig. 3). Expression

levels of all three genes were high in liver and kidney, whereas only *Pxmp2* was also highly expressed in heart tissue. Differential regulation has previously been shown for *Pxmp2* and *Pmp70* (*Pxmp1*) using peroxisome proliferater-induced or regenerating rat liver (Hashimoto et al., 1986; Hartl and Just, 1987; Lüers et al., 1990).

We analyzed the genomic structure and the chromosomal localization of the *Pxmp2*. The *Pxmp2* was found to consist of five exons within 11 kb of genomic DNA and was localized to the central region of mouse chromosome 5 at 59 cM. This region of mouse chromosome 5 contains a number of conserved loci that are represented on human chromosomes 6, 8, 12 and 22 (Mouse genome informatics at http://www.informatics.jax.org/reports/homologymap/mouse_human.shtml). A candidate gene for a human peroxisome related disease with unknown genotype was not found to map to any of these homologous regions.

Further studies including knockout of the gene are necessary to reveal the function and the mechanisms of regulation of the *Pxmp2* gene.

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