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Primary Structure of Human PMP69, a Putative Peroxisomal ABC-Transporter

Andreas Holzinger,[1] Stefan Kammerer, and Adelbert A. Roscher
Dr. v. Hauner Children's Hospital, Department of Clinical Chemistry and Biochemistry, Ludwig-Maximilian-University, Lindwurmstrasse 4, 80337 Munich, Germany

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We have cloned the cDNA of a novel human ABC-half-transporter highly similar to peroxisomal ABC-half-transporters such as the adrenoleukodystrophy protein (ALDP) and the peroxisomal protein 70 (PMP70). This 2927-bp cDNA codes for a 606 aminoacid (68.6 kDa) protein that was designated PMP69 (putative peroxisomal membrane protein). PMP69 is ubiquitously expressed. Transcript variants resulting from alternative polyadenylation and splicing events including one that confers an alternative C-terminus have been found. The PMP69 gene is localized on chromosome 14q24.3. ABC-half-transporters require a partner ABC-half-transporter to constitute a functional complex, either as a homodimer or a heterodimer. Defects in the gene coding for ALDP are the cause of adrenoleukodystrophy, a demyelinating disorder of the nervous system with strikingly varying clinical courses. PMP70 was implicated in the pathogenesis of a subgroup of Zellweger syndrome, a heterogenous group of peroxisome assembly disorders. PMP69 might be a heterodimer partner for one of these proteins, thus playing a role in modifying the clinical course of ALD or, alternatively, in peroxisome biogenesis.

ATP-binding-cassette (ABC) transporters are a family of integral membrane proteins involved in the transport of a variety of substrates across biological membranes (1,2). Mammalian examples include the cystic fibrosis transmembrane conductance regulator (CFTR), the multiple drug resistence proteins (MDR1 and MDR2), the transporters of antigen processing (TAP1 and TAP2) and others. To date, three members of the family have been located within the peroxisomal membrane: the adrenoleukodystrophy protein (ALDP) (3), the peroxisomal protein 70 (PMP70) (4), and the adrenoleukodystrophy related protein (ALDR) (5). Defects in the ALD gene are the underlying cause of adrenoleukodystrophy, a demyelinating disorder of the central nervous system with a variety of clinical manifestations. ALDP is a putative transporter of activated very long chain fatty acids (VLCFA) into the peroxisome, the exclusive site of β-oxidation of VLCFA. Mutations in the PMP70 gene have been associated with some forms of Zellweger syndrome, a heterogenous group of peroxisome assembly disorders (6,7). The function of ALDR is unknown. All three known peroxisomal ABC-transporters conform to the model of an ABC-half-transporter, requiring a partner half-transporter molecule (either as a homodimer or a heterodimer) to form a functional transporter. In *S. cerevisiae* two peroxisomal ABC-transporters (Pat1 and Pat2) have been demonstrated to associate with each other and to be involved in the import of VLCFA (8-10). Since the completion of the yeast genome project did not reveal more than these two peroxisomal ABC-half-transporters, peroxisomal transport mechanisms involving members of this protein family appear to be more complex in higher eukaryotes.

In order to elucidate the existence of additional peroxisomal ABC-transporters which may interact with known members of this protein family, we performed homology searches for ALDP and PMP70 related sequences in a database of expressed sequence tags (ESTs). We found such sequences and isolated the cDNA of a novel human ABC-half-transporter (putative peroxisomal membrane protein of 69 kDa, PMP 69). In this article we report the cDNA sequence and

[1] Correspondence should be addressed to Andreas Holzinger, M.D. Fax: (49) 089 51604192. E-mail: holzinger@kk-i.med.uni-muenchen.de.

Sequence data from this article have been deposited at the NCBI/Genbank Data Library under Accession number AFOO9746. Accession numbers of all sequences referred to in this article are derived from this database.

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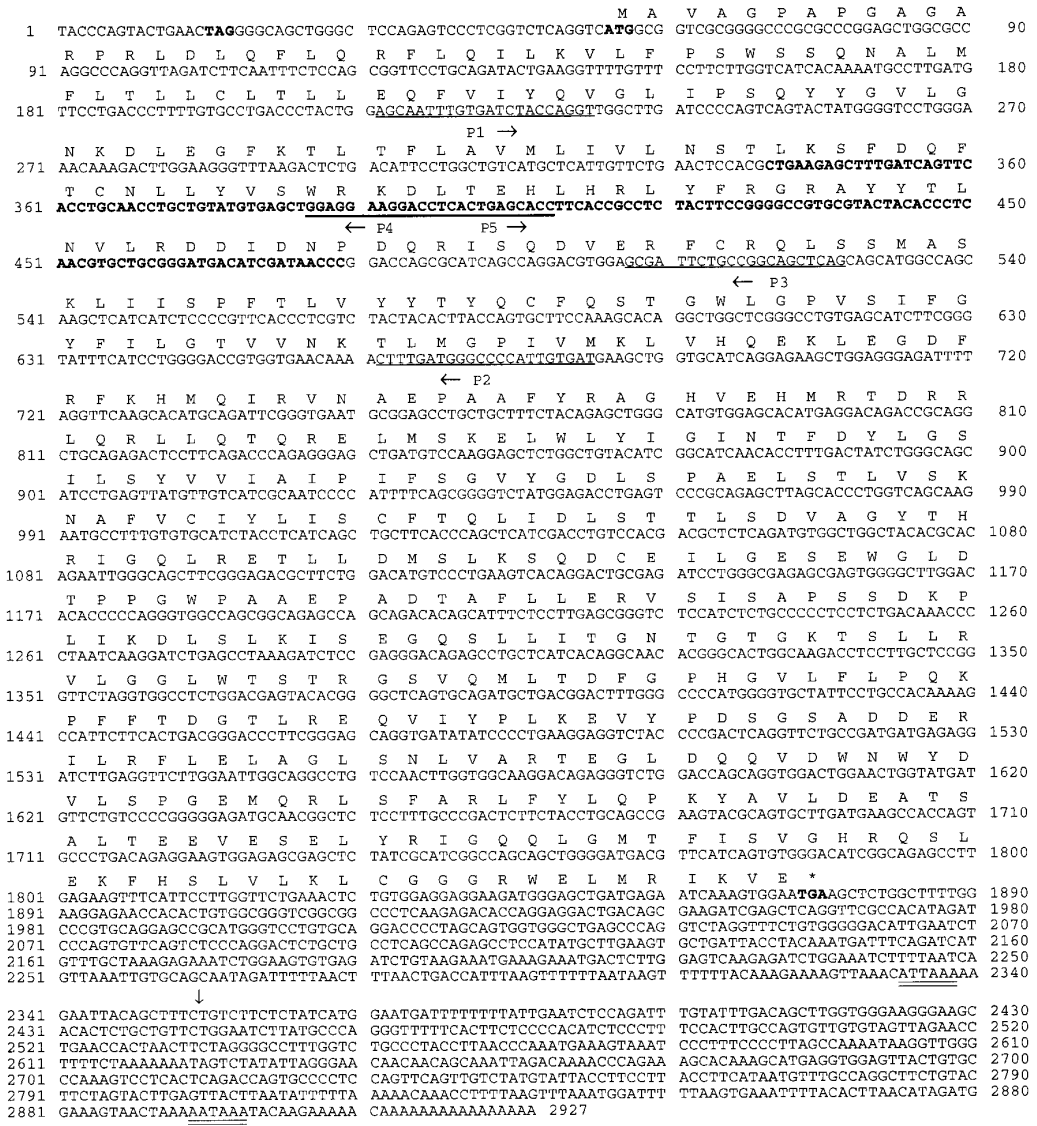

FIG. 1. cDNA and deduced aminoacid sequence of PMP69. The alternatively spliced 140-bp exon as well as the initiation and termination signals are indicated by bold letters. The vertical arrow indicates the upstream predominant polyadenylation site. Putative polyadenylation signals are indicated by double underlining, primer binding regions by single underlining. Primer directions are indicated by horizontal arrows.

the deduced primary protein structure as well as the chromosomal localization of the corresponding gene. Furthermore we present data on tissue distribution of mRNA expression, transcript variants, homology to ABC-transporters from man and other species and computer-algorithm-based characterization of PMP69.

MATERIALS AND METHODS

EST-search and cDNA cloning. The EST database dbEST (11) was searched for cDNA sequences encoding proteins similar to ALDP and PMP70 using the tblastn program (NCBI, National Center of Biotechnology Information, Washington, USA). EST entries W92104 and H50978 from the WashU-Merck Human EST Project were found and a 793-bp PMP69 specific probe was amplified from random primed human fibroblast cDNA with primers derived from the ends of the overlapping EST sequences. A human liver and spleen cDNA library was screened with this probe. Random-primed [α-^{32}P]dATP labelling and hybridization was performed by the Screening Service of the German Human Genome Project (12) following standard procedures and one clone (ICRFp512N2360Q2) was isolated and sequenced. The EST clones were obtained through the German Human Genome Project Ressource Center (Berlin, Germany) and sequenced. The 5′ end of the cDNA, not present in any of the above clones, was amplified from a human leukocyte cDNA library (5′ STRETCH, Clontech) using a vector primer and 5′ reverse gene specific primer. Additionally, adaptor-ligated human liver cDNA (Marathon PCR-ready cDNA, Clontech) was used to amplify DNA fragments con-

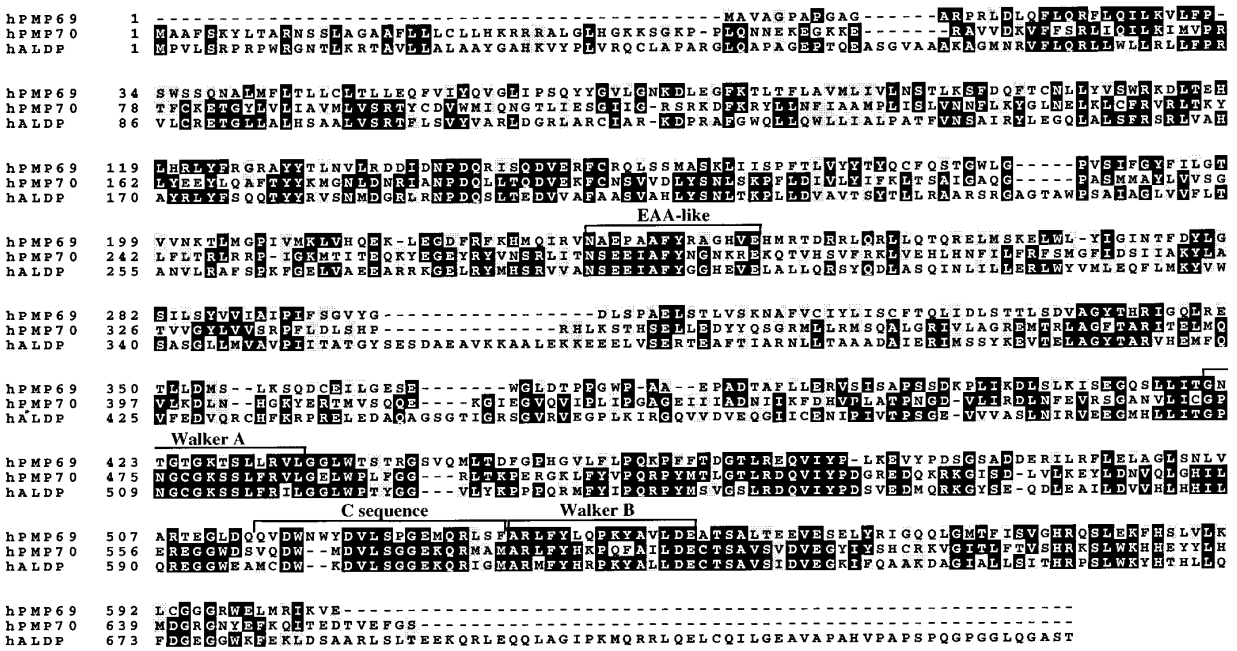

FIG. 2. Alignment of PMP69 with PMP70 and ALDP. Walker A and B motifs as well as the C-sequence and the EAA-like motif are indicated.

taining the 5′ cDNA end. PCR products were cloned and sequenced and a consensus sequence from at least 5 overlapping clones was obtained. Fluorescent dye terminator DNA sequencing was performed on an ABI 377 Sequencer.

Alternative splicing analysis. Sequencing of EST clones and PCR amplification of cDNA fragments revealed a number of splice variants. Using random-primed cDNA derived from total RNA the presence of these variants was examined by PCR in a variety of human tissues. Analysis of the exon-intron character was performed by PCR from human genomic DNA isolated from leukocytes.

TABLE 1

Evidence for Expression of PMP69 in Various Tissues and Cells

Tissue/cell types	Evidence
Pancreas	N
Kidney	N
Sceletal muscle	N
Liver	N, RT
Heart	N, RT, EST (W72656, W76618)
Brain	N, EST(F07011, F06287, F02255, T08166, Z39185, F09055)
Cerebellum	EST (AA322282)
Colon	RT
Lung	N, RT, EST(T89319)
Thymus	RT
Uterus	RT
Testis	EST (AA300616, T18936)
Placenta	N, RT
Skin fibroblast	RT, EST (Z21904)
Leukocyte	cDNA cloned from library
Retina	EST (W28686)

Note. N, Northern blot analysis; RT, reverse transcriptase-PCR; EST, PMP69-specific expressed sequence tag entry (NCBI GenBank accession No.) from specific tissue libraries.

Northern blot analysis. A human multiple tissue Northern blot containing 2μg of polyA$^+$ RNA per lane was purchased from Clontech. The same [α-^{32}P]dATP labelled probe that had served for cDNA library screening was used to detect PMP69 mRNA. Hybridization was performed by a modified method of Church and Gilbert (13) as described elsewhere (14).

Alignments and computer-assisted characterization. Protein alignments and computing of similarity scores were performed with the Align-program at ERIEE (Nimes, France). Database searches for homologous proteins were performed using the blastp program from NCBI. Hydrophobicity plots were generated according to Kyte and Doolittle with a window size of 12 aminoacids using the MacDNAsis computer program (Hitachi). Prediction of transmembrane helices was performed using the PhDtopology program at EMBL, Heidelberg.

RESULTS

Cloning of PMP69 cDNA and Characteristics of the Deduced Protein

We have cloned a 2927-bp cDNA with an open reading frame of 1818 bp (Fig. 1). Northern blot analysis (Fig. 3) reveals a single 2.5-kb signal. The size of the detected transcript corresponds to the sequence resulting from the utilization of the upstream polyadenylation signal (2355 bp). The difference between the length of the cloned cDNA and the specific signal in Northern blot analysis is attributable to the polyA tail. Therefore, the cloned cDNA seems to be full length.

TABLE 2

Sequence Similarities between PMP69 and Close Homologs

	Human PMP69	*C. elegans* 1418480	*Synech. sp.* 1001688	Human PMP70	Mouse ALDR	Human ALDP	Pat2 (*S. cerev.*)
Human PMP69							
C. elegans 141840	37.5						
Synech. sp. 1001688	30.3	26.9					
Human PMP70	28.4	24.4	27.0				
Mouse ALDR	26.1	25.0	26.6	36.8			
Human ALDP	25.2	23.9	24.2	36.2	63.1		
Pat2 (*S. cerevisiae*)	24.2	25.4	24.2	27.0	31.3	29.8	
Pat1 (*S. cerevisiae*)	20.2	19.2	21.0	24.7	29.6	27.4	23.1

Note. Percentage values of identical aminoacids of proteins homologous to PMP69 are given. NCBI GenBank accession No. are used to identify open reading frames from *C. elegans* and *Synechocystis sp.* The mouse ALDR sequence was included since the corresponding human cDNA has only been partially cloned to date.

The first initiation codon is preceded by a termination signal such that no other upstream start codon can be utilized. This cDNA encodes a 68.6 kD protein fulfilling the criteria of the ABC-transporter family of membrane proteins. Conserved motifs among ABC-transporters (15) such as the Walker A and Walker B sequence as well as the C-sequence could be localized in the deduced aminoacid sequence (Fig. 2). The EAA-like motif, a conserved region shared by prokaryotic and peroxisomal ABC-transporters (16), could also be identified. Using computer-assisted structure prediction the protein revealed to contain one transmembrane domain (consisting of six transmembrane helices) and one hydrophilic domain (data not shown) consistent with predictions for other peroxisomal ABC-transporters (16). A database search for homologous eukaryotic proteins revealed known human and yeast *peroxisomal* ABC-transporters to be the closest relatives. Alignments with PMP70 and ALDP demonstrated these proteins to be the closest *human* homologs (28.4% and 26.1% respectively of identical aminoacid residues, Table 1). Additionally, four highly homologous *C. elegans* open reading frames were found including No.1418480, the possible *C. elegans* ortholog of PMP69. Furthermore, striking similarity was found to prokaryotic proteins from *Synechocystis sp.* (No.1001688) (Table 2), *Mycobacterium tuberculosis* (No.1483552, 27.4% aminoacid identity) and *Haemophilus influenzae* (No.1075044, 26.7% identity; No.1175812, 25.1% identity). The levels of sequence similarity of these proteins with PMP69 are significantly higher than with all other human ABC-transporters.

Tissue Distribution of PMP69 Expression

Evidence for ubiquitous expression of PMP69 is presented by virtue of Northern blot analysis (Fig. 3), RT-PCR (Fig. 4) and PMP69-specific ESTs found in libraries derived from a variety of human tissues (Table 2).

Analysis of Transcript Variants

Alternative polyadenylation. Sequencing of the initially identified ESTs revealed two alternative polyadenylation sites resulting from polyadenylation signals as indicated in Fig. 1. Utilization of the downstream polyadenylation signal increases the transcript size by 560 bp.

Alternative transcript arising from exon skipping. PCR-amplification from cDNA with primers P1 and P2 yielded the expected 476-bp product but also a 336-bp

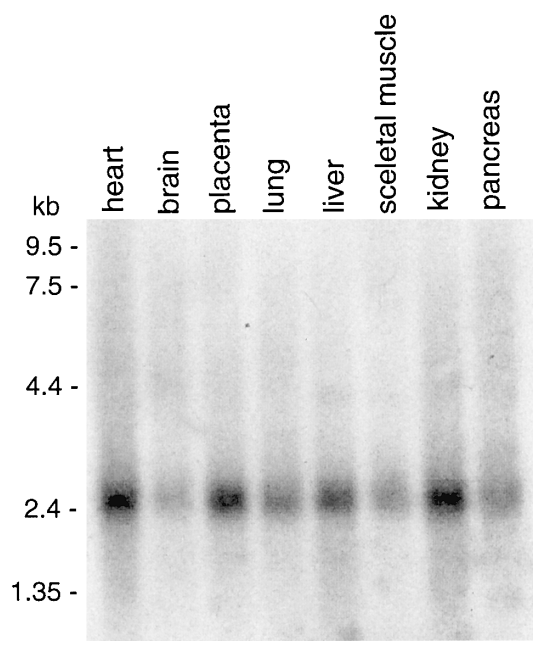

FIG. 3. Northern blot analysis. Using a 793-bp PMP69-specific cDNA probe, a single band of approximately 2.5 kb was detected in all tissues examined. This band corresponds to the transcript resulting from utilization of the upstream polyadenylation signal.

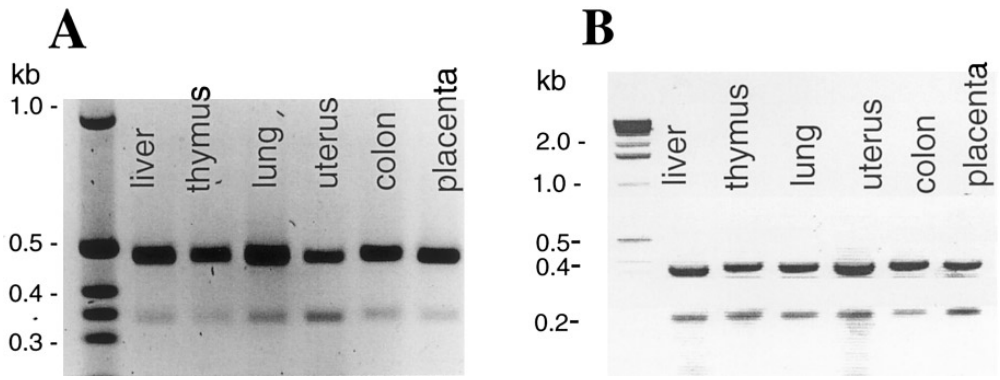

FIG. 4. PCR-analysis of splice variants. (A) PCR-amplification from random-primed cDNA with primers P1 and P2. The splice variant resulting from exon skipping (represented by the 336-bp product) is present in all tissues examined. (B) PCR-amplification from random primed cDNA with primers P6 and P7. The splice variant resulting from retaining an intron in the transcript (represented by the 372-bp product) is present in all tissues examined. Primer positions are indicated in Fig.1 and Fig.5.

product missing a 140-bp fragment (bp 430-479) (Fig. 1). The band corresponding to the alternative transcript was detected in all tissues examined (Fig. 4A). Analysis of the 5′ and 3′ ends of a 3.1-kb genomic amplification product with primers P1 and P3 revealed splice sites and intronic sequences precisely at the ends of the missing fragment. Analysis of genomic PCR products generated with primer combinations P1/P4 and P5/P3 revealed that the 140-bp fragment corresponds to a single exon flanked by a 1.6-kb intron at the 5′ end and a 1.5-kb intron at the 3′ end.

Alternative transcript arising from retaining an intron. Fig. 5 shows a 171-bp intron that is retained on a portion of transcripts. Using RNA from a variety of human tissues RT-PCR amplification with the primer combination P6/P7 demonstrated the presence of the alternative transcript (reflected by the 372-bp product) in all tissues examined (Fig. 4B). PCR from a genomic DNA template with the same primer combination yielded the 372-bp product only which confirmed the intronic character of the 171-bp fragment. Translation of this transcript would result in an alternative C-terminus of the PMP69 protein (Fig. 5).

Assignment of the Chromosomal Localization of the PMP69 Gene

The dbEST database entry N65934 (Sharma et. al., unpublished) a 227-bp transcript fragment identical to PMP69. This fragment was derived from cDNA selection experiments and shown to be part of a Yac contig localized on chromosome 14q24.3

DISCUSSION

By virtue of ESTs corresponding to an ABC-transporter (17) we have cloned the cDNA of a novel human

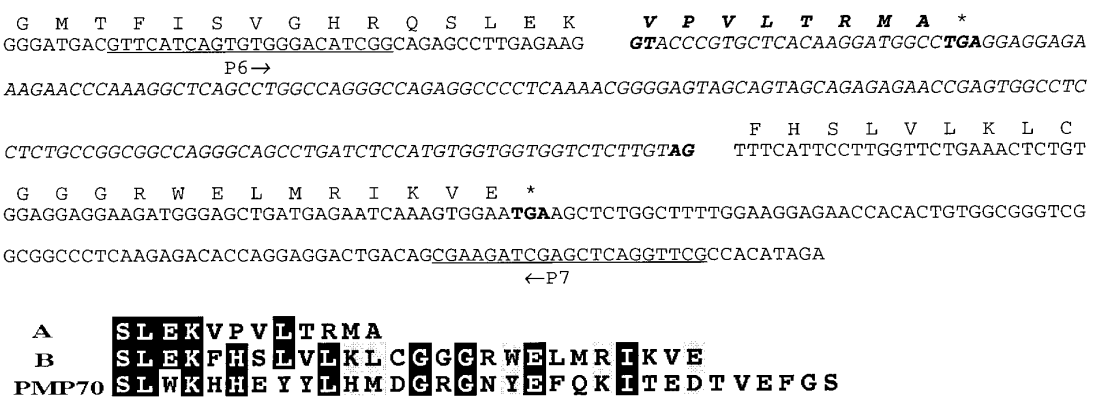

FIG. 5. Translational consequence of the splice variant resulting from retaining an intron in the transcript. The inclusion of the 171-bp intron (indicated by *italics*) in the transcript confers an alternative C-terminus (A, also shown in bold letters) that shows much weaker similarity to PMP70 than the translation product from the transcript not including this intron (B). Primer binding sites are indicated by underlining.

membrane protein (PMP69) that—by homology—belongs to the subgroup of ABC-transporters localized within the peroxisomal membrane. PMP70 is the closest known human relative. PMP69 appears to be ubiquitously expressed. A PMP69 cDNA fragment was assigned to chromosome 14q24.3. This finding, however, does not exclude the existence of further gene copies throughout the human genome.

Several transcript variants were detected. The transcript size seen on Northern blot (2.5 kb) corresponds to the transcript resulting from the upstream polyadenylation site (Fig. 1). The alternative polyadenylation transcript appears to be rare. The transcript variant resulting from a skipped 140-bp exon is probably not functional since the reading frame is altered. The transcript resulting from retaining the 171-bp intron confers an alternative C-terminus. Either of these transcript variants resulting from alternative splicing events are present in all tissues examined (Fig. 4). A functional role of the transcript conferring the alternative C-terminus cannot be excluded with certainty. It is, however, obvious that the C-terminus of the translation product displays similarity to PMP70 only if the 171-bp fragment is absent from the transcript (Fig. 5).

The PMP69 sequence is strikingly similar to the *C. elegans* open reading frame No. 1418480 which appears to be the ortholog. Given the similarity to prokaryotic proteins from *Synechocystis sp.* and other species it is intriguing to speculate that PMP69 might be an ancestor of other peroxisomal ABC-transporters.

Subcellular localization experiments of PMP69 have not been included in this study. If PMP69 is in fact localized wirhin the peroxisomal membrane, it is the fourth human member of the subgroup of peroxisomal ABC-half-transporters known to date. These molecules are expected to form either homodimers or heterodimers to constitute a functional transporter. The way peroxisomal ABC-transporters dimerize is not known nor are the substrates that are transported.

PMP69 might possibly be of importance in already recognized peroxisomal diseases. The clinical course of adrenoleukodystrophy is strikingly varying even within families ranging from lethal childhood cerebral to clinically asymptomatic forms (18). Secondary genetic factors may explain this phenomenon and changes such as polymorphisms in a heterodimer partner protein may contribute. If PMP69 reveals to be a heterodimer partner of ALDP it is a candidate for a modifier gene of the clinical course of adrenoleukodystrophy even if it may not be the exclusive partner. Since PMP70 has been implicated in some forms of Zellweger syndrome, PMP69—as a potential heterodimer partner—might alternatively play a role in peroxisome biogenesis.

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