cDNA cloning and tissue-specific expression of the phosphatidylcholine transfer protein gene

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We have isolated a cDNA containing the complete coding sequence of bovine liver phosphatidylcholine transfer protein (PC-TP). The deduced amino acid sequence consists of 213 amino acid residues and is, except for a lysine instead of an arginine at position 167, identical to the sequence determined by Edman degradation [Akeroyd, Moonen, Westerman, Puyk and Wirtz (1981) Eur. J. Biochem. **114**, 385–391]. A cDNA encoding amino acid residues 41–214 of mouse lung PC-TP was also isolated. The predicted amino acid sequence was 90% similar (81% identical) to the corresponding sequence of bovine liver PC-TP, demonstrating that PC-TP is conserved among mammalian species. By Southern blot analysis, evidence was obtained for the presence of a single bovine PC-TP-encoding gene. The expression of the PC-TP gene was determined during mouse embryonic development and in adult mouse tissues using an RNase protection assay. PC-TP RNA was present in embryos at all stages of development as early as the embryonic stem cell, suggesting a role for PC-TP in cell growth and differentiation. Towards the end of embryonic development, just before term, high levels of PC-TP RNA were found in the liver. This level was even higher 7 days post-term. In addition to adult liver, high levels of PC-TP RNA were also found in kidney and testis. The prominent presence of PC-TP in developing and adult liver is compatible with its proposed role in bile formation.

INTRODUCTION

Phospholipid transfer activity was initially discovered in the membrane-free cytosol of rat liver [1]. This activity is due to a number of different phospholipid transfer proteins, the most prominent of which is the phosphatidylcholine transfer protein (PC-TP). This protein was purified to homogeneity from bovine and rat liver [2,3]. In agreement with its absolute specificity for PC, the purified bovine PC-TP was found to contain one molecule of non-covalently bound PC [4]. Bovine and rat PC-TPs have a molecular mass of about 28 kDa, as determined by SDS/PAGE [5]. However, the isoelectric points of rat and bovine PC-TPs are different (i.e. 8.4 and 5.8 respectively), and antibodies raised against either protein are not cross-reactive with the other [5]. This suggests that rat and bovine PC-TPs are substantially different [6]. The complete primary structure of bovine PC-TP has been determined by automated Edman degradation. The protein consists of a single polypeptide chain of 213 amino acid residues, with two disulphide bridges at Cys^{17}–Cys^{63} and Cys^{93}–Cys^{207} [7]. Nothing is yet known about the primary structures of PC-TPs from other mammals.

The phospholipid transfer activity of PC-TP has been investigated extensively *in vitro*, but little is known about the function of PC-TP *in vivo* [6]. High concentrations of PC-TP are found in liver and lung [8]. In the lung PC is secreted as part of the lung surfactant. Concomitant with the increased production of surfactant in the fetal lung towards term, the levels of PC-TP are maximal just before term, in parallel with the levels of enzymes required for PC synthesis [8]. This is compatible with a role for PC-TP in surfactant production. The liver secretes large amounts of PC into the bile. Smit et al. [9] have shown that this process requires the P-glycoprotein encoded by the mouse *Mdr*2 gene. This P-glycoprotein is thought to act as a PC translocator, flipping PC from the inner to the outer leaflet [9–12]. PC-TP may contribute to this process by transporting PC from its site of synthesis, the endoplasmic reticulum, to the inner leaflet of the canalicular membrane [9]. The stimulation of PC-TP-mediated transfer of PC between membranes by submicellar concentrations of the common bile salts [13] is compatible with this postulated role of PC-TP in PC export from the liver.

To obtain more conclusive information on the physiological role of PC-TP, we have cloned cDNAs for bovine and mouse PC-TPs and used these clones to study the gene multiplicity and the expression of the gene during mouse development and in adult mouse tissues.

MATERIALS AND METHODS

Materials

Oligonucleotides were synthesized by Pharmacia (Uppsala, Sweden). Taq DNA polymerase and Moloney Murine Leukemia Virus reverse transcriptase were obtained from Gibco BRL (Gaithersburg, MD, U.S.A.). The pGEM-T kit was from Promega (Madison, WI, U.S.A.). The bovine liver λMAX cDNA library and the mouse lung λZAP cDNA library were from Clontech (Palo Alto, CA, U.S.A.) and Stratagene (La Jolla, CA, U.S.A.) respectively. [α-^{35}S]dATP, [α-^{32}P]dATP and [α-^{32}P]CTP were obtained from Amersham International (Amersham, U.K.).

RNA isolation

Bovine tissue was obtained from the local slaughterhouse (Kouwenhove B. V.). Mouse tissue was isolated from laboratory

Abbreviations used: PC, phosphatidylcholine; PC-TP, phosphatidylcholine transfer protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TAE, 40 mM Tris/acetate, 1 mM EDTA, pH 8.0.

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The cDNA sequences described in this paper have been deposited in the EMBL Nucleotide Sequence Database under the following accession numbers: bovine liver PC-TP cDNA (nucleotides −68 to 1435), Z50026; mouse lung PC-TP cDNA (nucleotides 119 to 645), Z50024; rat liver PC-TP cDNA (nucleotides 298 to 536), Z50023.

Table 1 Nucleotide sequences of the degenerate PCR primers and the corresponding amino acid sequences of bovine PC-TP

Oligonucleotides PCE536 and PCE575 are antisense to their amino acid peptides. In nucleotide sequences, R = A or G; Y = C or T; D = A, G or T; and N = A, C, G or T.

Primer	Nucleotide sequence	Amino acid sequence
PCB235	AAR CAR TGG GAY CAR TAY GT	Lys^{79}-Gln-Trp-Asp-Gln-Tyr-Val^{85}
PCB298	TAY TGG CAR GTN AAR TAY CC	Tyr^{100}-Trp-Gln-Val-Lys-Tyr-Pro^{106}
PCE536	GG RTT RTC RAA RTA RTA CAT	Met^{173}-Tyr-Tyr-Phe-Asp-Asn-Pro^{179}
PCE575	GC NGC CCA RTT DAT NAC CCA	Trp^{186}-Val-Ile-Asn-Trp-Ala-Ala^{192}

mice (129/Ola and FVB). Total RNA from mouse, rat and bovine tissues was isolated by an acidic guanidinium isothiocyanate/phenol/chloroform procedure [14]. Bovine liver poly(A)$^+$ RNA was isolated from total bovine liver RNA using the mRNA isolation kit from Qiagen (Hilden, Germany).

Isolation of a PC-TP cDNA fragment

First-strand cDNA was synthesized from 10 μg of total bovine liver RNA using the Stratagene First Strand Synthesis kit, with a random hexanucleotide mixture as primers. The reaction was carried out in a final volume of 50 μl according to the instructions supplied with the kit. A 2.5 μl sample of the reverse transcriptase reaction mixture was used as a template for PCR.

A PC-TP cDNA fragment was amplified from the bovine liver first-strand cDNA using a nested PCR with degenerate oligonucleotides. The degenerate oligonucleotides PCB235, PCB298, PCE536 and PCE575 were designed based on the known amino acid sequence of bovine PC-TP [7] (Table 1). The first PCR was performed with the primers PCB235 and PCE575 at final concentrations of 4.0 and 10.0 μM respectively. The PCR mixture contained 2.5 μl of the reverse transcriptase reaction mixture, the primers, 1.5 mM $MgCl_2$ and 2.5 units of Taq polymerase in Taq DNA polymerase buffer [50 mM KCl, 10 mM Tris/HCl, pH 9.0 at 25 °C, 0.1 % (w/v) gelatin and 1 % Triton X-100]. The final volume was 50 μl. The mixture was overlayed with 50 μl of paraffin oil. PCR was carried out in a PCR thermocycler (Perkin-Elmer) and the temperature profile consisted of 4 min at 94 °C, 20 cycles of 1 min at 94 °C, 2 min at 50 °C and 1 min at 72 °C, and finally 5 min at 72 °C. Aliquots of 10 μl of the PCR reaction mixtures were analysed by electrophoresis on a 1 × TAE (40 mM Tris/acetate, 1 mM EDTA, pH 8.0)/1.5 % agarose gel and visualized by ethidium bromide staining to identify the PCR products. The amplification did not give a detectable product, except for the band caused by the primers (Figure 1, lane 1). We therefore used an aliquot of the PCR mixture in a second PCR with nested primers. A 3 μl portion of the first PCR mixture was used in the second PCR with the primers PCB298 and PCE536 at final concentrations of 8.0 and 4.0 μM respectively. The second PCR (40 cycles instead of 20) amplified a DNA fragment of approx. 240 nt (Figure 1, lane 2). This PCR product was cloned into the pGEM-T vector according to the instructions supplied with the kit (Promega). The amplified cDNA fragment was sequenced by double-strand sequencing, using the dideoxy chain-termination method [15]. It had a length of 238 nt and encoded amino acid residues 100–178 of bovine PC-TP. The amino acid sequence of this peptide was identical to that previously determined by Edman degradation [7] except for the amino acid at position 167, which is a lysine instead of an arginine.

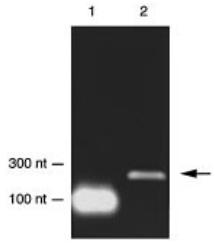

Figure 1 Electrophoretic analysis of the nested PCR products obtained by amplification of bovine PC-TP RNA

Lane 1, PCR product (10 μl), primers PCB235 and PCE575; lane 2, nested PCR product (10 μl), primers PCB298 and PCE536.

Cloning of a cDNA encoding bovine PC-TP

The 238 bp bovine liver PC-TP PCR fragment was used to screen a random oligonucleotide/oligo(dT) primed λMAX bovine liver cDNA library. The cDNA library was screened according to the manufacturer's protocol. The 238 bp PCR fragment was labelled with [α-^{32}P]dATP using the random primed labelling method [16]. Phages containing cDNA which hybridized with the probe were selected and isolated in successive cycles of purification. The phagemids present in the selected phages were excised according to the manufacturer's protocol. The inserts were sequenced using the double-strand sequencing method for identification. Initially about 0.5×10^6 plaques were screened, and a clone containing a 1.1 kb cDNA insert was isolated. This cDNA insert coded for amino acid residues 105–213 of bovine PC-TP. In addition a 3′-end non-coding sequence of about 800 nt was present. This 1.1 kb insert was used as a probe to screen another 0.5×10^6 plaques. A positive clone, with an insert of approx. 1 kb, was isolated which contained the complete coding sequence of PC-TP.

Cloning of a cDNA encoding murine PC-TP

For technical reasons we first amplified a segment of rat PC-TP cDNA from total rat liver RNA by using the PCR method described above. This PCR resulted in the amplification of a 238 bp rat PC-TP cDNA fragment (nucleotides 298–536; see the Results section). This cDNA fragment was used to screen a random oligonucleotide/oligo(dT) primed λZAP murine lung cDNA library. About 1×10^6 plaques were screened and one positive clone, with an insert of about 1.2 kb, was isolated.

RNA blot analysis

The complete coding sequence of bovine liver PC-TP was used to generate a probe for RNA blot analysis using the random primed labelling method. RNA blot analysis was performed according to Sambrook et al. [16]. Bovine liver poly(A)$^+$ RNA (10 μg) was separated on a 1 × TAE/1 % agarose gel containing 2.2 M formaldehyde, and transferred to a nylon membrane using the TurboBlot system (Schleicher & Schuell, Dassel, Germany). The membrane was further processed as described [16].

Southern blot analysis of bovine genomic DNA

Bovine genomic DNA was isolated from liver according to Laird et al. [17]. Approx. 15 μg of DNA was digested overnight with 25 units of restriction enzyme under conditions specified by the

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   -68
   TCCCGCCTCC GAACACGATC CCTGCCTGTC CCCTCTCCCT TCCGGGCCCC GGGGCGCCGC
-8       1/1                          22/8
TGCGAAGG ATG GAT CCT GGG GCC GGC GCC TTC TCG GAG GAG CAG TTC CGG GAG
         MET ASP PRO GLY ALA GLY ALA PHE SER GLU GLU GLN PHE ARG GLU
46/16                           70/24                  .
GCC TGC GCA GAG CTC CAG CGC CCC GCA CTC TCC GGG GCC GCC TGG GAG CTG
ALA CYS ALA GLU LEU GLN ARG PRO ALA LEU SER GLY ALA ALA TRP GLU LEU
97/33                           121/41
CTG GTG GAG ACC CAG GGC ATC AGC GTC TAC CGG CTG CTG GAC CAG CAG ACT
LEU VAL GLU THR GLN GLY ILE SER VAL TYR ARG LEU LEU ASP GLN GLN THR
148/50                          172/58
GGA TTG TAC GCG TAT AAG GTC TTT GGT GTT CTG GAG GAC TGC TTA CCA GAT
GLY LEU TYR ALA TYR LYS VAL PHE GLY VAL LEU GLU ASP CYS LEU PRO ASP
199/67                          223/75
CTG CTT GCA GAC GTC TAT ATG GAC TTA GCC TAT AGG AAA CAG TGG GAC CAA
LEU LEU ALA ASP VAL TYR MET ASP LEU ALA TYR ARG LYS GLN TRP ASP GLN
250/84                          274/92
TAT GTT AAA GAA CTC TAT GAA AAA GAA TGC AGT GGA GAA ACA GTG GTC TAC
TYR VAL LYS GLU LEU TYR GLU LYS GLU CYS SER GLY GLU THR VAL VAL TYR
301/101                         325/109
TGG CAA GTG AAG TAC CCT TTT CCC ATG TCT AAC AGA GAT TAT GTT TAT GTG
TRP GLN VAL LYS TYR PRO PHE PRO MET SER ASN ARG ASP TYR VAL TYR VAL
352/118                         376/126
CGG CAG CGG CAA GAG CTG GAC TTT GAA GGG CAG AAG GTC CAC GTG ATC CTG
ARG GLN ARG GLN GLU LEU ASP PHE GLU GLY GLN LYS VAL HIS VAL ILE LEU
403/135                         427/143
GCC CAG AGC ACC TCT GAG CCG CAG TTT CCA GAG AAG TCG GGT GTG ATC CGG
ALA GLN SER THR SER GLU PRO GLN PHE PRO GLU LYS SER GLY VAL ILE ARG
454/152                         478/160
GTG AAG CAC TAC AAG CAG AGG CTG GCG ATC CAG AGC GAT GGC AAG AAG GGG
VAL LYS HIS TYR LYS GLN ARG LEU ALA ILE GLN SER ASP GLY LYS LYS GLY
505/169                         529/177
AGC AAA GTT TTC ATG TAT TAC TTC GAT AAT CCA GGT GGC CAG ATT CCG TCC
SER LYS VAL PHE MET TYR TYR PHE ASP ASN PRO GLY GLY GLN ILE PRO SER
556/186                         580/194
TGG GTC ATT AAC TGG GCT GCT AAG AAT GGA GTT CCT AAC TTC TTG AAA GAC
TRP VAL ILE ASN TRP ALA ALA LYS ASN GLY VAL PRO ASN PHE LEU LYS ASP
607/203                         631/211                  652
ATG GTG AAA GCC TGT CAG AAC TAC AAG AAA ACC TAG GGGAGGAAGC
MET VAL LYS ALA CYS GLN ASN TYR LYS LYS THR AMB
653
TGGGAGCTTT  GTGTCCATGG  GATGCTGTTC  CTTAGTGCCA  CGGCCCCCGA  TTGCTCAGCC
TGCCTTCCTT  TCTGGCTCAG  AGGCCTGCAC  ACTCTCTAGC  ACTTGTCCCG  AGCGTGTTTG
CTGACATCTG  CTCCTTTCCC  AGTCTCCCCA  CCCCGGACCC  ACTGCCTTCC  TCCACTCTGG
AATGTGGGTT  CAGATTATAG  AAACCTTTGT  TTCTTTAAAC  AAAGAAACCT  TTGTTTAAAC
CTTTGTTCTT  AAAAAGTGGA  ATCCTCAGTA  GGGAACGCAG  TCCTTCCATT  GTGCCATTCT
AGGGGAAGGC  TGGGTGGAGG  GCTGACAACC  CTGCCTAAGA  GGGACTGATC  CCAGTCAGCT
GGCTCCTTCC  ACGTGTGAGT  TGTTGCAACC  TGCGAGGGTG  ATTCCTGCTG  AAGTTGCATT
GCTACGGAGC  TGACTGTCAT  CGCTCCAAGA  CTGGAAGACT  CATGTCCATT  TGCCTTATGC
TTTGCTGACT  TGGATTGCCT  AATTTGAGAT  AATCGTCCAC  TAAGTCCCTG  CCCATTCCTA
GTTGGGGAGG  ATCATAGGCT  GTTTTGATTT  CAAACTATTT  TGAAAAACAT  TGCAGGCCTA
ACTGCGTAGT  ATGATAACAA  TTTTTTTCTC  ATTATCCATG  TTCGGCTTTT  TCTTTTTCCC
ATATGCCCCT  GGGGAAGGCT  GTCCCACCTC  ACTAGCTCTG  CATTTTACTG  GACACTGAGG
CATCAAGGCA  ACTGTAGCAG  TTAACAGAAA  GCTGCTTTGC  AACTATAATT  GAGCTAAAAT
GAC 1435
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Figure 2 Nucleotide and deduced amino acid sequence of the 1500 bp cDNA encoding bovine PC-TP

The deduced amino acid sequence is identical to the previously determined amino acid sequence [7] except for amino acid residue 167, which is lysine instead of arginine.

manufacturer. Overnight electrophoresis was performed on a 0.6 % agarose gel, run in 1 × TAE buffer. Subsequently the DNA was transferred to a nitrocellulose membrane (0.1 mm pore size; Schleicher & Schuell). To generate a randomly primed probe, a 850 bp *Sma*I–*Pme*I fragment of the bovine PC-TP cDNA clone was used which contains almost the entire coding sequence plus part of the 3′ untranslated region. Hybridization was performed overnight at 63 °C. The blot was washed at 65 °C, twice in 2 × SSC/0.5 % SDS, and twice in 0.5 × SSC/0.1 % SDS.

RNase protection assay

The procedure for the RNase protection assay was that described by Zinn et al. [18] as modified by Baas et al. [19]. To generate a suitable template for generation of the RNase protection probe, the partial cDNA encoding murine PC-TP was used in a PCR with the primers PCB235 and PCE575. The conditions were as described above, except that the PCR consisted of 40 cycles and the template consisted of DNA from approx. 1000 phages, which contained the 1.2 kb cDNA insert encoding murine PC-TP. The resulting 341 bp PC-TP PCR fragment (mouse PC-TP coding sequence; positions 235–575 relative to the tentative translation start codon) was cloned into the pGEM-T vector as described above. To synthesize the murine PC-TP antisense RNA probe, the plasmid template was linearized with *Nco*I (in the pGEM-T polylinker) and transcription was performed with SP6 RNA polymerase. As a control for RNA recovery in the RNase protection assay a murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antisense probe was included. This probe was transcribed with SP6 RNA polymerase from *Bst*EII-linearized pmGAP ([20]; provided by Dr. J. K. Heath, Department of Biochemistry, University of Oxford, Oxford, U.K.) The labelled antisense RNA probes were incubated with 10 μg of total RNA from the tissue of interest. The protected antisense RNA probe

fragment was visualized by electrophoresis through a denaturing 5% polyacrylamide gel, followed by autoradiography.

RESULTS

Cloning of a cDNA encoding bovine PC-TP

We have used the known amino acid sequence of bovine PC-TP to design degenerate oligonucleotide primers for the amplification of a segment of the corresponding cDNA (Table 1). With nested primers we were able to amplify a 238 bp segment of bovine PC-TP cDNA. This segment was used to isolate a λMAX bovine liver cDNA clone with a cDNA insert corresponding to full-length PC-TP. The 1503 bp sequence contained a single open reading frame coding for the amino acid residues of bovine PC-TP (Figure 2). The canonical AATAAA polyadenylation site or poly(A) tail is absent from the cDNA, suggesting that part of the 3′ end of the mRNA is missing from our clone. This cDNA had no sequence similarity to sequences found in the EMBL Databank (see below).

The amino acid sequence deduced from the full-length cDNA is identical to the amino acid sequence determined by Akeroyd et al. [7] except for the residue at position 167, which is lysine instead of arginine. This lysine residue was also found in the 238 bp PC-TP PCR fragment. Since the two PC-TP cDNAs have been isolated from different bovine livers, and since the sequence analysis by automated Edman degradation clearly identified an arginine residue at position 167, we presume that the difference is due to a polymorphism. After completion of our work we noticed a recent entry into the EMBL Databank of a bovine liver cDNA clone (accession no. U21660) by D. E. Cohen and R. N. Green (Brigham and Women's Hospital, Harvard Medical School, Boston, MA, U.S.A.). This clone contains arginine at position 167.

On an RNA blot of bovine liver, the 1 kb bovine cDNA insert detected a single 1.9 kb band (Figure 3). This indicates that the cloned sequence lacks about 400 nt of the untranslated regions of the PC-TP mRNA. Since the polyadenylation site and the poly(A) tail are absent from the cloned sequence, it is likely that part of the 3′ untranslated region is missing.

Cloning of a cDNA encoding mouse PC-TP

Using the same PCR method as for bovine PC-TP, we isolated a 238 bp segment of rat PC-TP. This 238 bp cDNA fragment is 83% identical to the bovine PC-TP cDNA sequence (nucleotides 298–536; Figure 4, upper panel). The 238 bp rat PC-TP fragment was used to isolate a clone with an approx. 1200 bp insert from a λZAP murine lung cDNA library. The insert contained part of the coding sequence for murine lung PC-TP (nucleotides 119–645, relative to the tentative translation start codon) and the stop codon (Figure 4, upper panel). In addition, a 700 nt 3′-non-coding sequence was identified containing the canonical AATAAA polyadenylation signal and a poly(A) tail (results not shown). The murine PC-TP cDNA coding sequence has an identity of 81% with the bovine PC-TP cDNA sequence. The deduced amino acid sequence (residues 41–213) is 90% similar (81% identical) to bovine PC-TP (Figure 4, lower panel). Note that mouse PC-TP contains an additional amino acid at position 211, i.e. a histidine (Figure 4). No sequence homologies were

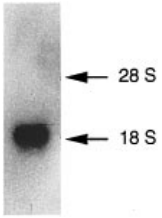

Figure 3 RNA blot analysis of bovine liver RNA with a PC-TP cDNA probe

A 10 μg sample of bovine liver poly(A)$^+$ RNA was hybridized with labelled PC-TP cDNA. The 1 kb cDNA clone containing the complete coding sequence of bovine PC-TP cDNA (nucleotides −68 to 1000 of the bovine PC-TP cDNA sequence) was labelled by random priming. No other bands were visible on the autoradiogram even after prolonged exposure.

```
                                119                                 150
bovine PC-TP                    C GTCTACCGGC TGCTGGACCA GCAGACTGGA
murine PC-TP                    . A......... ......... ..CA.G....

          151                                                       200
bovine PC-TP    TTGTACGCGT ATAAGGTCTT TGGTGTTCTG GAGGACTGCT TACCAGATCT
murine PC-TP    C.T..T.A.. .......G.. C.....C... ..A.G..... CG..G.C...

          201                                                       250
bovine PC-TP    GCTTGCAGAC GTCTATATGG ACTTAGCCTA TAGGAAACAG TGGGACCAAT
murine PC-TP    C..C.....T ..T..C.... ......A... C..A.... A ........G.

          251                                                       300
bovine PC-TP    ATGTTAAAGA ACTCTATGAA AAAGAATGCA GTGGAGAAAC AGTGGTCTAC
murine PC-TP    ....G..... ......C..G ..G..GA.TG A..A.C.G.T G....CA...
rat PC-TP                                                      ..T

          301                                                       350
BOVINE PC-TP    TGGCAAGTGA AGTACCCTTT TCCCATGTCT AACAGAGATT ATGTTTATGT
MURINE PC-TP    ...G..... .......... C..GC....C .......... .C..C..CAC
RAT PC-TP       ...C..... .......... C..TC....C .......... .T..C..CAC

          351                                                       400
BOVINE PC-TP    GCGGCAGCGG CAAGAGCTGG ACTTTGAAGG GCAGAAGGTC CACGTGATCC
MURINE PC-TP    C..C.....C .G...C.... .TG.G..CA. .AG....A.. T.....G...
RAT PC-TP       C..C.....G .G...C.... .TG.G..CG. .AG....A.. T.....G...

          401                                                       450
BOVINE PC-TP    TGGCCCAGAG CACCTCTGAG CCGCAGTTTC CAGAGAAGTC GGGTGTGATC
MURINE PC-TP    .......... T.T...G.CA ..T....... .C........ T..G..C...
RAT PC-TP       .........A T.T...T.TA ..T....... .G........ T..G..A...

          451                                                       500
BOVINE PC-TP    CGGGTGAAGC ACTACAAGCA GAGGCTGGCG ATCCAGAGCG ATGGCAAGAA
MURINE PC-TP    ..A.....A. .G.....A.. ...C...... ...G....T. .C........
RAT PC-TP       ..A.....G. .G........ ...C...... ...G....C. .T........

          501                                                       550
BOVINE PC-TP    GGGGAGCAAA GTTTTCATGT ATTACTTCGA TAATCCAGGT GGCCAGATTC
MURINE PC-TP    ........G. .......... .C.....T.. ...C..G... .....A....
RAT PC-TP       ........G. .......... .C.....T.. ...C..

          551                                                       600
BOVINE PC-TP    CGTCCTGGGT CATTAACTGG GCTGCTAAGA ATGGAGTTCC TAACTTCTTG
MURINE PC-TP    ........C. .......... ..A..C.... .......... A.....T...

          601                                                  645
BOVINE PC-TP    AAAGACATGG TGAAAGCCTG TCAGAACT-- -ACAAGAAAA CCTAG
MURINE PC-TP    ..G....... .......G.. ........AC C......... ....A
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          41                                                        80
bovine PC-TP    VYRLLDQQTG LYAYKVFGVL EDCLPDLLAD VYMDLAYRKQ
murine PC-TP    I......PS. ..E....... .G.S.A.... .....D....

          81                                                       120
bovine PC-TP    WDQYVKELYE KECSGETVVY WQVKYPFPMS NRDYVYVRQR
murine PC-TP    .......... ..SDEQM.A. .E......L. ......T...
rat PC-TP                            . .Q......L. ......T...

          121                                                      160
bovine PC-TP    QELDFEGQKV HVILAQSTSE PQFPEKSGVI RVKHYKQRLA
murine PC-TP    RD..VDRR.I Y.V...SI.A .......... ...Q...S..
rat PC-TP       RD..VDGR.I Y.V...NI.V .......... ...Q...S..

          161                                                      200
bovine PC-TP    IQSDGKKGSK VFMYYFDNPG GQIPSWVINW AAKNGVPNFL
murine PC-TP    .E.......R .......... ......L... ..........
rat PC-TP       .E.......R ........

          201         214
bovine PC-TP    KDMVKACQNY -KKT
murine PC-TP    .......... H...
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Figure 4 Alignment of the nucleotide and the deduced amino acid sequences of bovine, mouse and rat PC-TPs

Upper panel, nucleotide sequences; lower panel, amino acid sequences.

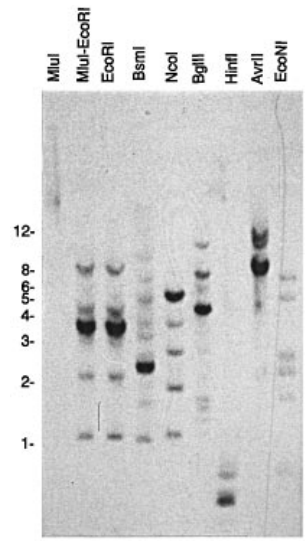

Figure 5 Southern blot analysis of genomic DNA

Digests of 15 μg of genomic DNA were size fractionated on a 1 × TAE/0.6% agarose gel and hybridized with a randomly primed 850 bp *Sma*I–*Pme*I fragment of bovine PC-TP cDNA, containing most of the coding sequence. Positions of size markers (kb) are shown on the left.

found in the EMBL Databank for the coding sequence. However, the 3′-non-coding nucleotide sequence contains one fragment of 130 nt which is highly similar (90 %) to the ubiquitous repetitive DNA sequence B1 found in the mouse genome [21] (results not shown).

Multiplicity of PC-TP genes

To test whether there is one or more genes, Southern blots of bovine genomic DNA were hybridized with an 850 bp cDNA probe containing a major part of the coding sequence and a small part of the 3′-untranslated region of the PC-TP mRNA. Figure 5 shows that digestion by *Eco*RI, *Bsm*I, *Nco*I, *Bgl*II and *Hinf*I yielded one major hybridizing band. Two very large bands were obtained by digestion with *Avr*II. These results strongly suggest that a single gene encodes PC-TP. The additional faint bands in the digests may be interpreted to indicate that the gene is distributed over several exons.

Tissue distribution of PC-TP

The tissue distribution of mouse PC-TP was investigated with an RNase protection assay using a mouse PC-TP specific probe. In each analysis a probe for GAPDH RNA detection was included as an internal control for RNA loading and integrity. The mouse PC-TP antisense probe, generated by *in vitro* transcription of the 341 nt mouse PC-TP cDNA fragment, had a length of 380 nt (Figure 6A). A second fragment of about 440 nt was present,

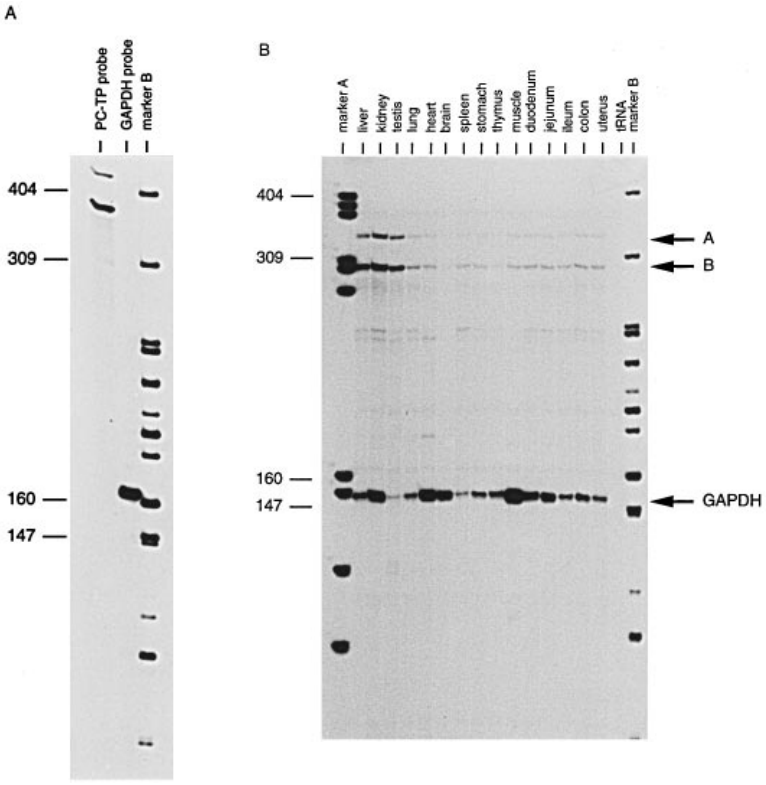

Figure 6 Determination of PC-TP RNA levels in tissues of adult mice by RNase protection

Total RNA (10 μg) was hybridized to ^{32}P-labelled antisense RNA probes (PC-TP and GAPDH), treated with RNase A and size-fractionated on a 5% polyacrylamide gel. The protected GAPDH RNA band serves as an internal control for RNA loading and integrity. Tissues containing PC-TP RNA yielded not only the full-length protected fragment (A) but also an additional protected product (B). Together the two products represent the total PC-TP RNA in the sample. The positions of these fragments are indicated by the arrows on the right-hand side of (**B**). The sizes of the marker bands are indicated on the left, in nucleotides: marker A, MP19 labelled fragments, generated by digestion with *Dde*I; marker B, pBR322 labelled fragments, generated by digestion with *Msp*I.

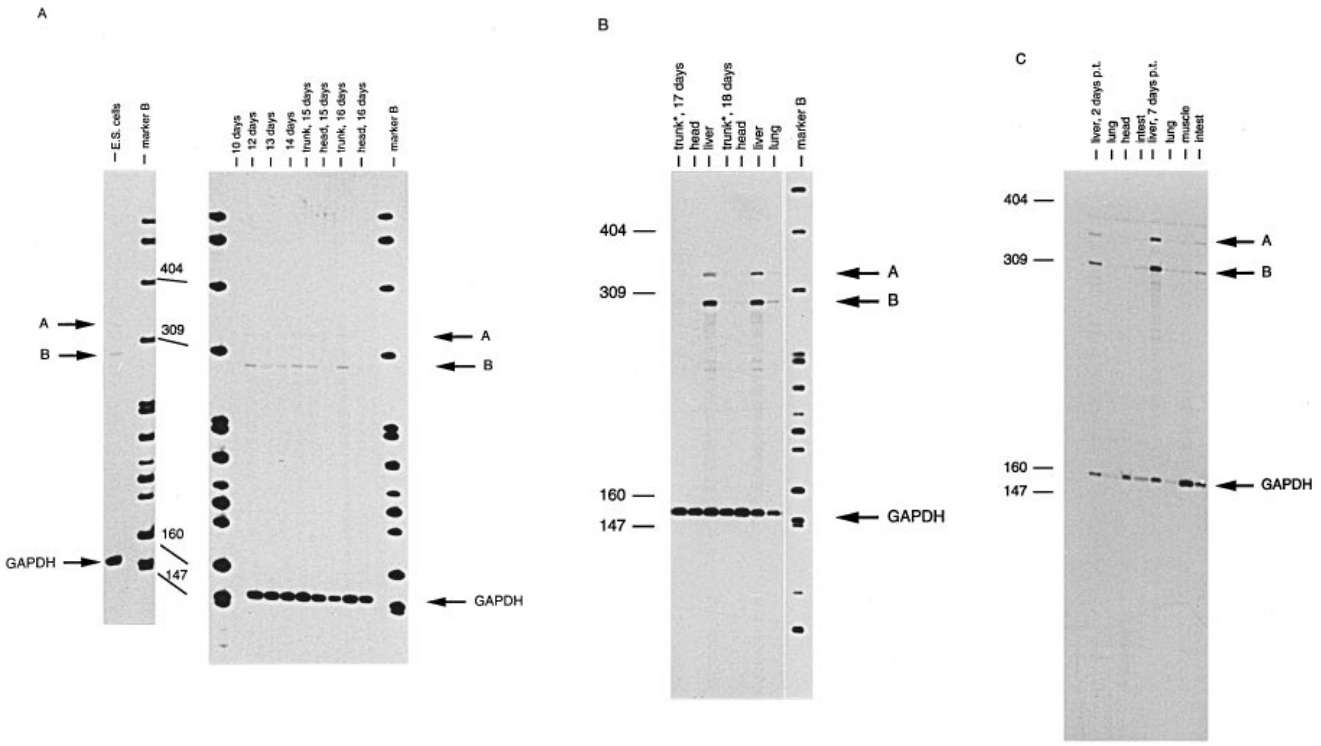

Figure 7 Determination of PC-TP RNA levels in tissues of fetal and postnatal mice by RNase protection

See the legend of Figure 6 for experimental details. (**A**) and (**B**) show expression in embryonic tissues at various stages of gestation, as indicated above the lanes, and in embryonic stem (E.S.) cells. (**C**) Expression in mouse tissues at 2 and 7 days post-term (p.t.); intest, intestine.

probably the result of incomplete linearization of the vector. The GAPDH antisense probe had a length of 160 nt (Figure 6A). Both antisense probes were completely digested by RNase A in the absence of complementary RNA (Figure 6B, tRNA lane). Incubation of the antisense probe with total RNA extracted from various tissues of mature mice followed by digestion with RNase A yielded one 150 nt protected fragment representing GAPDH, and two fragments (300 and 340 nt) representing PC-TP (Figure 6B). The probe used in the RNase protection assay was generated by PCR with the degenerate primers PCB235 and PCE575 and subsequently cloned in pGEM-T. Sequencing showed that the probe contained some nucleotides in the primer regions which may affect the efficient hybridization of these regions with the mouse PC-TP mRNA (results not shown). We therefore presume that due to these mismatches the RNase A digestion gives rise to two fragments, i.e. the complete 340 nt fragment and a smaller 300 nt fragment. The observed variation in GAPDH RNA levels in the various tissues agrees with the difference in GAPDH expression in the tissues [22].

As shown in Figure 6(B) the level of PC-TP RNA is high in kidney, liver and testis and low in the other tissues tested. PC-TP RNA was hardly detected in brain and thymus. The high PC-TP RNA level in kidney and liver was also detected in bovine tissues using an RNase protection assay with a bovine PC-TP probe (results not shown). By measuring the PC-TP level in different rat tissues using a specific radioimmunoassay, Teerlink et al. [8] obtained comparable results except that intermediate levels of PC-TP were found in kidney and spleen.

Since the function of PC-TP is still a matter of conjecture, we determined at what stage of mouse embryonic development expression of the PC-TP gene starts. As shown in Figure 7(A), PC-TP RNA is present in whole embryos as early as 10 days of gestation. PC-TP RNA is even detectable in embryonic stem cells (cell line E14 derived from 129/Ola mice). The level of PC-TP RNA remained constant during development up to 16 days of gestation. On days 15 and 16 the head and trunk were analysed separately, showing that PC-TP RNA is present mainly in the trunk. By days 17 and 18 of gestation PC-TP RNA is found predominantly in the liver, is clearly detectable in the lung but is hardly present in the head (Figure 7B). These data show that the PC-TP gene is expressed throughout the development of the mouse embryo. Post-term, PC-TP RNA is present predominantly in the liver; the level at day 7 has increased relative to that at day 2 (Figure 7C).

DISCUSSION

In this study we describe the isolation of cDNAs encoding bovine liver PC-TP and segments of rat and mouse PC-TPs. A previous comparison of rat and bovine liver PC-TPs suggested that these proteins are distinctly different. Their isoelectric points were 8.4 and 5.8 respectively, and polyclonal antibodies raised against either protein did not cross-react with the other [5]. The sequence analyses presented here, however, show a considerable degree of sequence conservation between bovine and rodent PC-TPs (around 80% amino acid identity). This high conservation of the primary structure has also been observed for the mammalian phosphatidylinositol transfer protein (99% identity between rat, mouse and human [23–25]) and the non-specific lipid transfer protein (Sterol Carrier Protein 2) (90% identity between mouse, rat and human [26–28]). The high degree of conservation in phospholipid transfer proteins in mammals suggests a strong

dependence of the function of these proteins on their primary structure.

As a carrier for PC, bovine PC-TP has a binding site for the *sn*-1- and *sn*-2-acyl chains of PC. Part of the binding site was identified as the extremely hydrophobic peptide Val^{171}–Phe^{176} [29], which was predicted to form an anti-parallel β-sheet with the segment Gln^{182}–Trp^{190} [30]. It is of interest that the amino acid residues contributing to this proposed binding site are completely conserved in the bovine and mouse PC-TP sequences, except for amino acid residue 187, which is a conservative replacement (Figure 4, lower panel).

Using the mouse PC-TP cDNA we determined PC-TP RNA levels in adult mouse tissues and during mouse embryonic development. PC-TP RNA was shown to be present in mouse embryos at all stages of development as early as the embryonic stem cells. This suggests a possible universal role for PC-TP in cell growth and differentiation. PC-TP may be essential for membrane biogenesis by delivering PC to sites of growth. PC-TP might also play a role in the signal transduction pathway which involves the receptor-controlled hydrolysis of PC by PC-specific phospholipases C and D [31,32]. In this pathway PC-TP may deliver PC from intracellular stores to sites of enzymic hydrolysis where PC is converted into lipid mediators such as phosphatidic acid, diacylglycerol and arachidonic acid, which are important for cell proliferation and differentiation. Such a function of PC-TP would be analogous to that of phosphatidylinositol transfer protein, which is an essential component in the phosphoinositide-based signal transduction pathway [33,34] and probably also in vesicular transport [35]. Previous studies have shown that *in vitro* PC-TP may catalyse a net transfer of PC to membranes devoid of this phospholipid [13,36–38].

Towards the end of embryonic development, just before term, high levels of PC-TP RNA were found in the liver. This high level was maintained after birth (2 days post-term) and rose to even higher levels at 7 days post-term. The prominent presence of PC-TP in the developing liver is compatible with its proposed role in bile formation, which begins shortly after birth [9,13,39]. In contrast to what was proposed on the basis of PC-TP activity [8,40], we found no correlation between lung PC-TP mRNA and the onset of lung surfactant synthesis. It should be noted, however, that surfactant is made by type II cells which constitute a small subfraction of the lung cells. Cytological analyses are required to determine whether the PC-TP mRNA levels correlate with surfactant secretion by these cells.

To secrete PC into bile, mammals employ a specialized class III P-glycoprotein translocator, encoded by the *Mdr*2 gene in mice, which is predominantly expressed in liver. In view of the proposed relationship between PC-TP and *Mdr*2 [9–12] we anticipated the existence of a specific PC-TP gene that is only expressed in liver. From our Southern blot analysis we infer, however, that PC-TP is encoded by a single gene which is differentially expressed in a great variety of mouse tissues, in embryos and even in embryonic stem cells. The isolation of PC-TP cDNA provides a tool with which to address the physiological function of this protein by genetic approaches, e.g. by generating mice in which the PC-TP gene has been disrupted.

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REFERENCES

1 Wirtz, K. W. A. and Zilversmit, D. B. (1968) J. Biol. Chem. **243**, 3596–3602
2 Kamp, H. H., Wirtz, K. W. A. and van Deenen, L. L. M. (1973) Biochim. Biophys. Acta **318**, 313–325
3 Lumb, R. H., Kloosterman, A. D., Wirtz, K. W. A. and van Deenen, L. L. M. (1976) Eur. J. Biochem. **69**, 15–22
4 Demel, R. A., Wirtz, K. W. A., Kamp, H. H., Geurts van Kessel, W. S. M. and van Deenen, L. L. M. (1973) Nature New Biol. **246**, 102–105
5 Poorthuis, B. J. H. M., van der Krift, T. P., Teerlink, T., Akeroyd, R., Hostetler, K. Y. and Wirtz, K. W. A. (1980) Biochim. Biophys. Acta **600**, 376–386
6 Wirtz, K. W. A. (1991) Annu. Rev. Biochem. **60**, 73–99
7 Akeroyd, R., Moonen, P., Westerman, J., Puyk, W. C. and Wirtz, K. W. A. (1981) Eur. J. Biochem. **114**, 385–391
8 Teerlink, T., Van der Krift, T. P., Post, M. and Wirtz, K. W. A. (1982) Biochim. Biophys. Acta **713**, 61–67
9 Smit, J. J. M., Schinkel, A. H., Oude Elferink, R. P. J., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A. A. M., Ottenhoff, R., van der Lugt, N. M. T., van Roon, M. A., van der Valk, M. A., Offenhaus, G. J. A., Berns, A. J. M. and Borst, P. (1993) Cell **75**, 451–462
10 Oude Elferink, R. P. J., Offenhof, R., Vanwijland, M., Smit, J. J. M., Schinkel, A. H. and Groen, A. K. (1995) J. Clin. Invest. **95**, 31–38
11 Smith, A. J., Timmermans-Hereijgers, J. L. P. M., Roelofsen, B., Wirtz, K. W. A., van Blitterswijk, W. J., Smit, J. J. M., Schinkel, A. H. and Borst, P. (1994) FEBS Lett. **354**, 263–266
12 Ruetz, S. and Gros, P. (1994) Cell **77**, 1071–1081
13 Cohen, D. E., Leonard, M. R. and Carey, M. C. (1994) Biochemistry **33**, 9975–9980
14 Chomcynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
15 Sanger, F. and Coulson, A. R. (1975) J. Mol. Biol. **94**, 441–448
16 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Harbor Spring, NY
17 Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenish, R. and Berns, A. (1991) Nucleic Acids Res. **19**, 4293
18 Zinn, K., Diamaio, D. and Maniatis, T. (1983) Cell **34**, 865–879
19 Baas, F., Jongsma, A. P. M., Broxterman, H. J., Arceci, R. J., Housman, D., Scheffer, G. L. Riethorst, A., van Groenigen, M., Niewint, A. W. M. and Joenje, H. (1990) Cancer Res. **50**, 5392–5398
20 Rathjen, P. D., Nichols, J., Toth, S., Edwards, D. R., Heath, J. K. and Smith, A. G. (1990) Genes Dev. **4**, 2309–2318
21 Krayev, A. S., Kamerov, D. A., Skryabin, K. G., Ryskov, A. P., Bayev, A. A. and Georgiev, G. P. (1980) Nucleic Acids Res. **8**, 1201–1215
22 Piechaczyk, M., Blanchard, J. M., Marty, L., Dani, Ch., Panabieres, F., El Sabouty, S., Fort, Ph. and Jeanteur, Ph. (1984) Nucleic Acids Res. **12**, 6951–6963
23 Dickeson, S. K., Lim, C. N., Schuyler, G. T., Dalton, T. P., Helmkamp, G. M. and Yarbrough, L. R. (1989) J. Biol. Chem. **264**, 16557–16564
24 Geijtenbeek, T. B. H., de Groot, E., van Baal, J., Brunink, F., Westerman, J., Snoek, G. T. and Wirtz, K. W. A. (1994) Biochim. Biophys. Acta **1213**, 309–318
25 Dickeson, S. K., Helmkamp, G. M. and Yarbrough, L. R. (1994) Gene **142**, 301–305
26 Moncecchi, D., Pastuszyn, A. and Scallen, T. J. (1991) J. Biol. Chem. **266**, 9885–9892
27 Ossendorp, B. C., van Heusden, G. P. H., de Beer, A. L. J., Bos, K., Schouten, G. L. and Wirtz, K. W. A. (1991) Eur. J. Biochem. **201**, 233–239
28 Yamamoto, R., Kallen, C. B., Balalola, G. D., Rennert, H., Billheimer, J. T. and Strauss, J. F., III (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 463–467
29 Westerman, J., Wirtz, K. W. A., Berkhout, T., van Deenen, L. L. M., Radhakrishman, R. and Khorana, H. G. (1983) Eur. J. Biochem. **132**, 441–449
30 Akeroyd, R, Lenstra, J. A., Westerman, J., Vriend, G., Wirtz, K. W. A. and van Deenen, L. L. M. (1982) Eur. J. Biochem. **121**, 391–394
31 Exton, J. H. (1994) Biochim. Biophys. Acta **1212**, 26–42
32 Cockcroft, S. (1992) Biochim. Biophys. Acta **1113**, 135–160
33 Thomas, G. M. H., Cunningham, E., Fensome, A., Ball, A., Totty, N. F., Truong, O., Hsuan, J. and Cockcroft, S. (1993) Cell **74**, 1–20
34 Kaufmann-Zeh, A., Thomas, G. M. H., Ball, A., Prosser, S., Cunningham, E., Cockcroft, S. and Hsuan, J. J. (1995) Science **268**, 1188–1190
35 Liscovitch, M. and Cantley, L. C. (1995) Cell **81**, 659–662
36 Berkhout, T. A., Van den Bergh, C., Mos, H., De Kruijff, B. and Wirtz, K. W. A. (1984) Biochemistry **23**, 6894–6900
37 Nichols, J. W. and Pagano, R. E. (1983) J. Biol. Chem. **258**, 5368–5371
38 Wirtz, K. W. A., Devaux, P. F. and Bienvenue, A. (1980) Biochemistry **19**, 3395–3399
39 Sakamoto, H. and Akino, T. (1972) Tohoku J. Exp. Med. **106**, 61–74
40 Engle, M. J., van Golde, L. M. G. and Wirtz, K. W. A. (1978) FEBS Lett. **86**, 277–281