Cloning and Expression of a Novel Phosphatidylethanolamine N-Methyltransferase

A SPECIFIC BIOCHEMICAL AND CYTOLOGICAL MARKER FOR A UNIQUE MEMBRANE FRACTION IN RAT LIVER*

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Phosphatidylethanolamine N-methyltransferase catalyzes the synthesis of phosphatidylethanolamine from phosphatidylethanolamine and is most active in liver. A cDNA for this enzyme from a rat liver cDNA library has been cloned, sequenced, and expressed in COS-1 cells, McArdle-RH7777 rat hepatoma cells, and SF9 insect cells. The expressed protein was capable of converting phosphatidylethanolamine into phosphatidylcholine in intact COS-1 cells, which normally have very low methyltransferase activity. The calculated molecular mass of the methyltransferase protein is 22.3 kDa, which is equivalent to that of the pure protein isolated from rat liver. Comparison of the sequence of the cloned rat liver methyltransferase with the yeast phosphatidylethanolamine methyltransferase PEM2 gene product revealed 44% identical amino acids and 68% similarity in the two predicted protein sequences. A polyclonal antibody was raised against a synthetic peptide corresponding to the carboxyl-terminal region of the enzyme and was affinity purified. The antibody recognized a single protein with a molecular mass of approximately 20 kDa when either rat liver proteins or proteins derived from the transfected COS-1 cells were electrophoresed on polyacrylamide gels containing sodium dodecyl sulfate. Surprisingly, the antibody exhibited no reactivity with endoplasmic reticulum proteins, even though the major phosphatidylethanolamine methyltransferase activity resides on this subcellular organelle. Instead, the antibody specifically recognized a protein in a unique subcellular membrane fraction purified from a crude mitochondrial preparation on a Percoll gradient. Immunocytochemical examination by electron microscopy showed positive labeling only in unique regions of the hepatocytes. The data suggest that this phosphatidylethanolamine methyltransferase is a specific marker for this unique membrane fraction.

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Phosphatidylethanolamine N-methyltransferase (PEMT)† is an enzyme in animals that converts PE to PC and uses AdoMet as the methyl donor. The liver is the only mammalian organ with significant PEMT activity (Ridgway, 1989). The majority of PEMT activity is localized to the cytosolic surface of the ER (Ridgway, 1989). The enzyme was purified from rat liver in 1987 (Ridgway and Vance, 1987) and has been shown to exist as a single protein with a molecular mass of 19 kDa.

The major pathway for PC biosynthesis in all animal cells is the CDP-choline pathway (Vance, 1989), but Sundler and Åkesson (1975) have estimated that in rat liver between 20 and 40% of the PC is derived from the methylation of PE. The reason for the specific expression of PEMT in liver is not known. PEMT may simply provide an alternative to the CDP-choline pathway, or the enzyme may have an unknown function that is required by the liver. PEMT and PC are absent from most prokaryotes such as Escherichia coli but are found in yeast (Carman and Henry, 1989; Carman, 1989). The requirement of PC for eucaryotic life may relate to specialized eucaryotic features such as organelle function or signal transduction (Exton, 1990).

Genetic and cloning studies have demonstrated the existence of two genes in yeast that code for PEMT activity (Kodaki and Yamashita, 1987). PEM1 codes for an enzyme that has a molecular mass of 101,202 daltons and catalyzes only the first methylation step, i.e., the conversion of PE to phosphatidylmonomethylethanolamine. PEM2 encodes a smaller enzyme comprising 206 amino acids with a calculated molecular mass of 23,150 daltons. The PEM2 gene product catalyzes all three transmethylation reactions in the conversion of PE to PC but preferentially catalyzes the methylation of PMME and phosphatididymethylethanolamine (Gaynor and Carman, 1990).

Because PEMT is a liver-specific enzyme, we wished to define the elements that regulate its tissue-specific expression in animals. As a first step toward this goal, we initiated the cloning of the cDNA for PEMT. A rat liver cDNA library was screened and a positive clone, which coded for an enzyme with 199 amino acids that was homologous to the PEM2 gene product, was identified. Unexpectedly, when we screened subcellular fractions from rat liver with antibody to the carboxyl terminus of the protein, it failed to localize to the ER. Since the majority of PEMT activity is located on the ER (Vance

and Vance, 1988), this result is surprising. Instead of reacting with a protein of the ER, the antibody reacted specifically with a protein of a unique membrane fraction isolated on a Percoll gradient as a "contaminant" of a mitochondrial preparation (Vance, 1990). Several other key enzymes involved in lipid biosynthesis are enriched in this membrane fraction. The question of whether this fraction is a unique, defined entity or an artifact generated by tissue homogenization appears to have resolved as a result of the specific localization of the cloned PEMT to this subcellular membrane.

**Materials and Methods**

**cDNA Cloning and Sequencing**—The NH₂-terminal sequence (30 amino acid residues) of purified rat liver PEMT was obtained by Edman degradation (Ridgway, 1988). Two oligonucleotides (CTGCACTCTTGGTACCCCTGGCAAGGACCCCTCPTI and C7GACCATCCTACCTACACCCCTCAGTAATAATGT) were synthesized, and separate samples were subcloned for sequence analysis. All the cDNA clones were isolated and sequenced. The insert was 500 base pairs in length and was potentially a partial fragment of rat liver PEMT cDNA according to analysis of its open reading frame. An oligo(dT)-primed rat liver cDNA library was probed with this fragment. Among 20 million colonies screened, four positive clones were studied further.

Amino acid residues of the carboxyl terminus of rat liver PEMT, YRRKATRLHKRS, was synthesized, and separate samples were acrylamide gel method (Sanger et al., 1977). The rat liver cDNA library was probed with this fragment. Among 20 million colonies screened, four positive clones were studied further and subcloned for sequence analysis. All the cDNA clones were sequenced by LKB automated DNA sequencing, and the sequences in both directions were confirmed by the deoxyxigeneration/poly-acrylamide gel method (Sanger et al., 1977).

**Antibody Production**—A peptide corresponding to the predicted 12 amino acid residues of the carboxyl terminus of rat liver PEMT, YRRKATRLHKRS, was synthesized, and separate samples were coupled to keyhole limpet hemocyanin and bovine serum albumin by the Alberta Peptide Institute, Edmonton, Alberta, Canada. Rabbits were immunized with the keyhole limpet hemocyanin-peptide conjugates. The antibody was purified on an affinity column in which the antibody was coupled to CNBr-activated Sepharose 4B (Harlow and Lane, 1988).

**RNA Analysis**—Total rat liver RNAs were prepared by guanidine/CSCl gradient centrifugation (Chirgwin et al., 1979). Briefly, rat liver was thoroughly perfused with phosphate-buffered saline. The liver was then rapidly removed and homogenized with a Polytron homogenizer. The total RNA was pelleted by CsCl gradient centrifugation. mRNAs were isolated from the total RNA by oligo(dT) column purification (from Collaborative Research Inc. and using the recommended protocol). Oligo(dT) probes were labeled with [32P]P, at the 5'-end by Tdv DNA kinase (Harrison and Zimmerman, 1988). cDNA probes were labeled with [32P]P by random-primed labeling kit obtained from Life Technologies, Inc. Northern blots were performed according to a previously described procedure (Reed and Mann, 1985).

**Immunoblotting of Proteins**—Proteins in membranes were solubilized by boiling for 10 min in 1% SDS sample buffer, separated on a 12.5% polyacrylamide, 0.1% SDS gel and electrically transferred to a nitrocellulose membrane (Laemmli, 1970; Towbin et al., 1979). The membrane was incubated with affinity-purified PEMT-2 antibody (0.3 μg/ml) in 25 mm Tris/HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20 for 1 h. The second antibody, obtained from Sigma, was a 1:10,000 dilution of stock solution of anti-rabbit IgG linked to horseshadish peroxidase, and was added for 30 min. The protein was detected by the enhanced chemiluminescence assay using the procedure recommended by the manufacturer (Amersham Corp.). The membrane was exposed to the film for times ranging from 10 to 120 s.

**Expression of the Cloned cDNA**—COS-1 and RH-7777 cells were grown in a 5% CO₂ incubator at 37° C in Dulbecco's modified Eagle's medium that contained 5% fetal bovine serum (COS-1 cells) or 10% fetal bovine serum plus 10% horse serum (RH-7777 cells). A full-length cDNA that contained PEMT-2 was inserted into a PCMV-5 vector. The original cDNA fragment was inserted into the Smal site of pUC 18 during construction of the cDNA library (Kalmar et al., 1990). The rat liver cDNA library was screened with labeled oligonucleotides mentioned earlier, and positive plasmids were purified. The full-length fragment of the PEMT cDNA was subsequenced into multichloning sites of pTZ19R. EcoRI and HindIII sites of the cloning vector are unique for the plasmid carrying PEMT cDNA. EcoRI is located at the 5'-end of the PEMT open reading frame. The full-length PEMT cDNA was liberated from pTZ19R by EcoRI/HindIII digestion. The expression vector pCMV5 was a gift from Dr. David Russell (Department of Molecular Genetics, University of Texas, Southwestern Medical Center, Dallas, TX). The full-length PEMT cDNA fragment was then ligated into EcoRI and HindIII sites of the pCMV5 so that the 5'-end of the PEMT open reading frame was adjacent to the 3'-end of the CMV promoter. The resultant plasmid was purified twice by cesium chloride centrifugation. Routinely, 10 μg of purified plasmids were used for transfection.

**Expression in COS-1 Cells**—COS-1 cells were transfected by the DEAE-dextran procedure (Ashman and Davidson, 1985). RH-7777 cells were transfected according to the calcium phosphate precipitation method (Chee and Okayama, 1987). After 48 h the transfected cells were scraped off the dishes and disrupted by homogenization with a Dounce homogenizer in a buffer that contained 10 mm Tris/HCl (pH 7.2), 150 mm NaCl, 1 mm EDTA, followed by sonication with a probe sonicator for 10 s. Cell membranes were prepared by centrifugation of a 600 x g supernatant at 350,000 x g for 15 min. The pellet was washed and resuspended in homogenization buffer for 10 s. Twenty-five μg of protein were routinely used in PEMT enzyme assays (Ridgway and Vance, 1992). The enzyme activity of PEMT is expressed as nmoles of 1Hmethyl groups incorporated per min/mg of protein. Protein was assayed according to the Bio-Rad protocol (Bradford, 1976).

**Expression in Transfected COS Cells**—The expression of cloned PEMT in insect cells, the cDNA for PEMT was liberated from pTZ19R by EcoRI/PstI digestion. The baculovirus expression vector, PVL 1392, was cleaved with EcoRI and PstI, and the cDNA for PEMT was ligated into this plasmid. Complete description of baculovirus vectors and methods can be found in O'Kelly et al. (1992). The insect cell line, Sf9, was derived from Spinola S16656 and was a gift from Pharmingen. Sf9 insect cells were grown in TNM-FH medium supplemented to 10% with fetal bovine serum. Medium from the initial transfection was harvested, serially diluted, and assayed for the presence of recombinant virus by plaque formation. Five recombinant plaques were isolated from which the virus was eluted in 1 ml of medium. Five hundred μl of this virus solution was used to infect 3 x 10⁶ SF9 cells. Three days after infection, 1 ml of the culture medium containing the amplified recombinant viral population was used to infect 9 x 10⁶ SF9 cells to yield a working viral stock. Two ml of viral stock (~2 x 10⁶ plaque-forming units) were used to infect 5 x 10⁶ SF9 cells for a period of 3 days. The infected SF9 cells were washed twice with phosphate-buffered saline and harvested by scraping with a rubber policeman, flash-frozen in a dry ice/ethanol bath, and stored at -70 °C prior to assay for PEMT activity. The SB-expressed PEMT was used for all kinetic studies.

**Preparation of Subcellular Fractions of Rat Liver**—Female Sprague-Dawley rats (200-250g) were fed standard chow and water ad libitum. These rats were perfused with ice-cold phosphate-buffered saline through the portal vein and rapidly removed, and subcellular fractions (ER1 and ER2 fractions, Golgi membranes, plasma membrane, mitochondria and nuclei) were isolated according to modifications (Vance, 1980) of the procedure of Croze and Morre (1984). The ER1 fraction was isolated from the final discontinuous sucrose gradient at the interface between the sucrose solutions of 1.5 and 2.0 M, whereas the ER2 fraction was isolated from the interface between the sucrose solutions of 1.3 and 1.5 M from the same gradient. The isolated membrane fractions were assayed for the following marker enzymes: NADPH-cytochrome c reductase (ER) (Vance and Vance, 1988. UDP-galactose:N-acetylglucosamine galactosyltransferase (trans-Golgi) (Bergeron et al., 1973), 5'-nucleotidase (plasma membrane) (Howell and Palade, 1982), and cytochrome c oxidase (mitochondria) (Wharton and Tzagoloff, 1967). The purity of the fractions has been previously documented (Vance and Vance, 1988). After the procedure of Croze and Morre (1984), the cytochrome c oxidase activity of the mitochondrial fraction enriched in lysosomes was prepared (Vance, 1990) from livers of rats that had been treated with Triton WR-1339. Acid phosphatase (lysosomal marker enzyme) was assayed according to an established procedure (Vance, 1980). Peroxidases were isolated by the method of Padyek and Rieder (1966). Peroxidase from the peroxisomal marker enzymes, catalase (Baudhuin et al., 1964) and palmitoyl-CoA oxidase (Völk and Fahimi, 1985) were assayed according to published procedures.

**Crude Mitochondria**—Prepared either by the method described.
above or by an alternative method (Vance, 1990; Hovius et al., 1990), were subjected to a final purification step. The crude mitochondrial pellet was suspended in a solution containing 250 mM mannitol, 5 mM HEPES (pH 7.4), 0.5 mM EGTA, and 0.1% bovine serum albumin, and the suspension was layered on top of a solution containing 225 mM mannitol, 5 mM HEPES (pH 7.4), 0.1% bovine serum albumin, and 30% Percoll. The tubes were centrifuged for 30 min at 95,000 × g. A dense band containing purified mitochondria was removed from approximately two-thirds down the tube. Another membrane fraction, previously designated as fraction X (Vance, 1990), was isolated from the Percoll gradient as the white band immediately above the mitochondria. The band was collected and centrifuged at 6,300 × g for 10 min. The supernatant was centrifuged at 100,000 × g for 1 h, and the pellet was retained. All isolated membrane fractions, except mitochondria, were stored at −70 °C in buffer containing 0.25 M sucrose, 10 mM Tris/HCl (pH 7.4), and 0.1 mM phenylmethylsulfonyl fluoride. Mitochondria were stored at −70 °C in 250 mM mannitol, 5 mM HEPES (pH 7.4), 0.5 mM EGTA, and 0.1% bovine serum albumin. The protein content of all subcellular membrane fractions was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

**Isolation and Sequence of the cDNA for a Rat Liver PEMT**

The purification of PEMT from rat liver microsomes has been described previously (Ridgway and Vance, 1987). The purified protein was partially sequenced from the amino terminus by Edman degradation (Ridgway, 1988). Two degenerate oligonucleotide probes, coding for two different amino acid sequences, were designed by guessing the third nucleotide of each codon according to statistical preference (Maruyama et al., 1986; Lathe, 1985), were synthesized according to the NH₂-terminal sequence. A random-primed rat liver cDNA library was screened with the two probes. One cDNA fragment of 500 base pairs, recognized by both probes, was used to screen an oligo(dT)-primed rat liver cDNA library for the full-length cDNA of PEMT. A cDNA fragment, 913 nucleotides in length, was isolated. The sequence analysis of the cloned cDNA fragment revealed that the longest open reading frame, corresponding to 199 amino acid residues, was the potential coding region of the PEMT from rat liver (Fig. 1).

The predicted amino acid sequence at the NH₂ terminus of the open reading frame was in very good agreement with that of the purified protein, except that the first methionine residue was absent from the NH₂-terminal sequence of the purified protein. An in-frame stop codon sequence was found at position −63 from the first ATG of the open reading frame, confirming that the first ATG of the open reading frame could indeed be utilized to initiate translation (Kozak, 1991). The polyadenylation signal, AATAAA, occurred 180 nucleotides downstream from the stop codon of the putative PEMT and 15 nucleotides upstream from the poly(A) tail (Fig. 1). The above evidence strongly suggested that the clone was a full-length cDNA for rat liver PEMT.

The open reading frame encodes a 199-amino acid protein with a calculated molecular mass of 22.3 kDa. The calculated pKₐ of this protein is 8.3. Sixty-four percent of the residues are hydrophobic amino acids, and 36% are hydrophilic. The predicted protein sequence contains four hydrophobic domains at amino acids 13–35, 47–69, 86–108, and 160–182. The regions between the first and second hydrophobic domains (amino acids 36–46) and the carboxyl-terminal peptide (189–199) are each positively charged. Particularly striking is the basic character of the carboxyl-terminal peptide, which contains 4 arginines and 2 lysines.

Two PEMT genes are responsible for PC synthesis via PE methylation in yeast. These genes have previously been cloned by complementation of the corresponding mutations (Yamashita et al., 1982; Kodaki and Yamashita, 1987). Comparison of the rat liver PEMT sequence with the yeast PEM2 gene product (Fig. 2A) reveals a very significant similarity between the two sequences. Forty percent of the amino acid sequences are identical and 68% are similar in the two predicted protein sequences. Some similarity between the cDNA for PEMT and PEMI was also observed (Fig. 2A). The hydrophobic domains of both protein sequences (Fig. 2B) suggest the possibility of four transmembrane segments for both liver PEMT and yeast PEM2p.

**Functional Expression of the PEMT cDNA**—As confirmation that the cloned cDNA did indeed code for PEMT, the cDNA was expressed functionally in three different cell lines. We detected a 4–5-fold increase in the PEMT activity in a homogenate of the rat hepatoma cells, McA-RH7777 (Table 1). Surprisingly, endogenous PEMT activity in these cells was negligible compared to that in homogenates from normal rat hepatocytes, in which the specific activity was 1.17 nmol/min/mg protein. A 10-fold increase of activity was observed in the COS-1 cell expression system, which provided the advantages of high copy number replication of the plasmid and a strong promoter. The highest expression was achieved in the baculovirus-infected SF9 cells in which the specific activity of PEMT was 12 times higher than the PEMT activity in purified ER from rat liver (1.63 nmol/min/mg protein) (Vance and Vance, 1988).

The expression of PEMT was evaluated in COS-1 cells by immunoblots. A peptide was designed according to the translated sequence of the COOH terminus of the open reading frame of the cloned cDNA. A polyclonal antibody specific for this peptide was generated by immunization of a rabbit and the antibody was affinity-purified on a peptide-bovine serum albumin affinity column. As shown in Fig. 3, the anti-PEMT antibody recognized a single band of approximately 28 kDa when the proteins derived from the PEMT-transfected COS-1 cells were electrophoresed on polyacrylamide gels containing SDS. The PEMT protein was absent from cells transfected with the plasmid, pCMV, that lacked the PEMT cDNA insert.

Further evidence that the PEMT cDNA coded for PEMT activity was obtained from labeling experiments with transfected COS-1 cells. The cells were incubated with [²H]ethanolamine (which is rapidly incorporated into PE) for 60 min or with [methyl-²H]methionine for 24 h. The incorporation of label into PC was determined. A 6-fold increase in radioactivity in PC isolated from PEMT-transfected cells, compared with control cells, was observed when labeled ethanolamine...
was the precursor (Table II). When labeled methionine was the precursor, the increase was 3-fold. These results indicate that the expressed protein was capable of converting PE to PC in intact COS-1 cells.

Characterization of the Expressed PEMT Activity—Since no PEMT activity was detected in non-transfected Sf9 cells, the activity of the expressed enzyme in cell homogenates was characterized without interference by endogenous PEMT activity. In Table III, the properties of the cloned and expressed PEMT were probed with either the full-length cDNA or with enzyme was obtained by Northern blot analysis of the mesosomal fraction of the liver (Audubert and Vance, 1983). The activity of the expressed enzyme in cell homogenates was characterized without interference by endogenous PEMT activity on liver microsomes. When labeled methionine was the precursor, the increase was 3-fold. These results indicate that the expressed protein was capable of converting PE to PC in intact COS-1 cells.

Activity—Since no PEMT activity was detected in non-transfected Sf9 cells, the activity of the expressed enzyme in cell homogenates was characterized without interference by endogenous PEMT activity. In Table III, the properties of the cloned and expressed PEMT were probed with either the full-length cDNA or with enzyme was obtained by Northern blot analysis of the mesosomal fraction of the liver (Audubert and Vance, 1983). The activity of the expressed enzyme in cell homogenates was characterized without interference by endogenous PEMT activity.

Examination of the tissue distribution of PEMT with polyclonal antibody to the carboxyl-terminal peptide demonstrated that PEMT protein was only present in liver (Fig. 5). Even though lung had the second highest specific activity for PEMT, about 10% of the liver activity (Table IV), no PEMT protein could be detected (Fig. 5) even after prolonged exposure of the blot to x-ray film (data not shown). All other tissues examined had less than 3% of the specific activity of liver (Table IV). Possibly, an alternate form of the enzyme, not recognized by the antibody, was responsible for the PEMT activity in lung.

Subcellular Localization of PEMT—The highest specific activity of PEMT in rat liver was in the ER fractions (Fig. 6A). We therefore expected that the protein encoded by the cloned PEMT cDNA would be localized mainly to the ER. Subcellular fractions were prepared from rat liver, and the proteins of the membrane fractions were separated by electrophoresis on a 12.5% polyacrylamide gel containing 0.1% SDS. In immunoblots, the antibody recognized a major protein band of approximately 20 kDa in a liver homogenate (Fig. 6B). To our surprise, the 20-kDa protein recognized by the antibody was absent from both ER fractions but was exclusively present in the unique rat liver subcellular fraction (Percoll fraction.
**PE Methyltransferase: A Unique Membrane Marker**

Fig. 2. Protein sequence comparison of rat liver PEMT, yeast PEM2 and PEM1 gene products. Panel A, sequence comparison. Vertical lines indicate identical residues, and conserved amino acids are indicated by colons. The cloned PEMT is referred to as PEMT-2 under “Discussion.” Panel B, hydrophobicity profile comparison. The hydrophobicity profiles were analyzed by the Kyte and Doolittle (1982) method. The hydrophobicity value is given on the vertical axis, and the amino acid position is indicated on the horizontal axis. Rat liver PEMT is presented in the upper panel, and yeast PEM2 protein is given in the lower panel.

**Table I**

*Expression of PEMT in eucaryotic cells*

The PEMT activities were assayed according to the method described previously (Ridgway and Vance, 1992). PMME was used as the exogenous substrate for the reaction at pH 9.2. The expression of PEMT in all three systems is described under “Experimental Procedures.” The 600 x g supernatant from cellular homogenates was used for the PEMT activity assays. The controls are homogenates from cells transfected with pCMV without the PEMT cDNA insert.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Expression</th>
<th>PEMT activity (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH7771</td>
<td>Control</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>PEMT</td>
<td>128</td>
</tr>
<tr>
<td>COS-1</td>
<td>Control</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>PEMT</td>
<td>138</td>
</tr>
<tr>
<td>Sf9</td>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PEMT</td>
<td>20,409</td>
</tr>
<tr>
<td>Rat liver homogenate</td>
<td>Control</td>
<td>1,238</td>
</tr>
</tbody>
</table>

The membrane fraction, which was separated from mitochondria on a Percoll gradient, was examined for the presence of marker enzyme activities characteristic of ER (NADPH:cytochrome c reductase and glucose-6-phosphate phosphatase), Golgi (UDP-galactose:N-acetylglucosamine galactosyltransferase), mitochondria (cytochrome c oxidase), lysosomes (acid phosphatase), peroxisomes (acyl-CoA oxidase and catalase), and plasma membrane (5'-nucleotidase) (Vance, 1990). The specific activities of all these marker enzymes in the novel membrane fraction were very low relative to those activities in the organelles for which these enzymes are markers. The exceptions to this statement were NADPH:cytochrome c reductase and glucose-6-phosphate phosphatase, for which the activities were 25-30% and approximately 300%, respectively, of those in purified ER membranes. Notably, previous efforts in finding a specific marker protein for this unique rat liver fraction had been unsuccessful.

in Fig. 6B) that has been previously described and implicated in specialized lipid synthesis and transport processes (Vance, 1990).
The anti-PEMT antibody was highly specific for a 20-kDa protein in the homogenate (Fig. 6B). Minor bands of other molecular sizes were observed in some purified membranes. Minor cross-reaction was evident with a low molecular mass protein in cytosol that contains no PEMT activity (Fig. 6A). Since cross-reaction of the antibody with other proteins was minimal, thin sections of rat liver tissue were examined by immunogold electron microscopy. The results, shown in Fig. 7, are consistent with data obtained from the immunoblots. The goat anti-rabbit IgG probe reacted with anti-PEMT antibody located in clusters within the hepatocytes. Typically, four or five gold patches were found in one cell, frequently located in the vicinity of mitochondria. Consistent with the subcellular fractionation studies, no immunoreactivity was located in the vicinity of mitochondria. Consistent with the minor cross-reaction was evident with a low molecular mass molecular sizes were observed in some purified membranes. 

The anti-PEMT antibody was highly specific for a 20-kDa protein in the homogenate (Fig. 6B). Minor bands of other molecular sizes were observed in some purified membranes. Minor cross-reaction was evident with a low molecular mass protein in cytosol that contains no PEMT activity (Fig. 6A). Since cross-reaction of the antibody with other proteins was minimal, thin sections of rat liver tissue were examined by immunogold electron microscopy. The results, shown in Fig. 7, are consistent with data obtained from the immunoblots. The goat anti-rabbit IgG probe reacted with anti-PEMT antibody located in clusters within the hepatocytes. Typically, four or five gold patches were found in one cell, frequently located in the vicinity of mitochondria. Consistent with the subcellular fractionation studies, no immunoreactivity was located in the vicinity of mitochondria. Consistent with the subcellular fractionation studies, no immunoreactivity was observed with ER, mitochondria, or other identifiable organelles of the hepatocytes (Fig. 7, A-C). Studies in which the PEMT antibody was omitted showed no gold labeling within the hepatocytes (Fig. 7D). Fig. 7 shows that the antibody is bound to a unique membrane in the hepatocyte, which is not recognizable as any known subcellular structure.

**DISCUSSION**

The Cloned PEMT Is a Specific Marker for a Unique Membrane Fraction in Liver—The majority of PEMT activity in liver is present on ER membranes (Vance and Vance, 1988). This enzyme will hereafter be referred to as PEMT-1. We have found, however, that the protein product (hereafter referred to as PEMT-2) of the PEMT cDNA that we have cloned is located specifically on a unique membrane distinct from the bulk of the ER. The location of PEMT-2 was deduced by immunoblot analysis of isolated subcellular membrane fractions from rat liver and by immunogold electron microscopy studies of liver slices. According to the immunoblotting experiments, PEMT-2 was not present in either the light or the heavy ER fractions, nor was it present in other isolated organelle membranes such as plasma membrane, Golgi, nuclei, or mitochondria.

The PEMT-2-containing membrane, which was separated from mitochondria on a Percoll gradient, possesses some, but not all, of the properties of ER. For example, in this membrane the specific activity of a frequently used ER marker enzyme, NADPH-cytochrome c reductase, is only 25–30% of that in the ER itself. In contrast, the specific activity of an alternative ER marker enzyme, glucose-6-phosphate phosphatase, is approximately 3-fold higher in this membrane than in the ER (Vance, 1990). The specific activities of other marker enzymes such as those of mitochondria, Golgi, plasma membrane, lysosomes, and peroxisomes were very low (Vance, 1990). The unique PEMT-2-containing membrane isolated from the Percoll gradient is also enriched, compared with the ER, in several enzymes involved in lipid biosynthesis, such as phosphatidylinositol synthase (Vance, 1990), diacylglycerol acyltransferase, and acyl-CoA:cholesterol acyltransferase. In addition, several membrane-bound enzymes required for the biosynthesis of PC and PE, including PEMT activity, are present in the membrane with specific activities similar to, or higher than, those in the ER (Vance, 1990).

The function of this unique PEMT-2-containing membrane is not known. However, since this membrane is enriched in enzymes responsible for the biosynthesis of lipids, and since the membrane was isolated in association with mitochondria, an attractive speculation is that the membrane may provide a "bridge" that would facilitate the transfer of newly synthesized lipids to the mitochondria. Available experimental evidence supports the idea that transfer of newly made phosphatidylserine to the mitochondria occurs via a collision-based mechanism, perhaps involving contact between the mitochondria and the membrane in which phosphatidylserine is synthesized (Voelker, 1989a, 1989b; Vance, 1991). Phosphatidylserine is decarboxylated to PE only in mitochondria (Dennis and Kennedy, 1972). Thus, the PEMT-2-containing membrane may also be involved in transferring PE out of mitochondria so that phosphatidylserine-derived PE can be methylated to PC. In addition, apolipoproteins B and E, which are constituents of nascent very low density lipoprotein particles, are abundant in the PEMT-2-containing membrane fraction (Vance, 1990). Because the PEMT-2-containing membrane fraction is enriched in glucose-6-phosphate phosphatase and lipoproteins, perhaps this fraction has an important role in secretion from hepatocytes.

Additional evidence that the isolated membrane is indeed a specific entity within the cell has been provided by immu-
**Table III**

Comparison of expressed PEMT with microsomal activity from rat liver

Values for the pH optimum and apparent $K_{m}$ for AdoMet in rat liver microsomes were taken from Audubert and Vance (1983).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH optimum</th>
<th>Apparent $K_{m}$ for AdoMet</th>
<th>Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microsomes</td>
<td>Sf9 cells</td>
<td>Microsomes</td>
</tr>
<tr>
<td>PE</td>
<td>58</td>
<td>ND*</td>
<td>0.28</td>
</tr>
<tr>
<td>PMME</td>
<td>10.2</td>
<td>10.0</td>
<td>65</td>
</tr>
<tr>
<td>PDME</td>
<td>96</td>
<td>ND*</td>
<td>2.34</td>
</tr>
</tbody>
</table>

* ND, not determined.

The activity was linear with protein concentration when PE and PMME were substrates. In the case of PDME, the relationship was sigmoidal so that at a protein concentration of 170 mg/ml the specific activity was 9.8 nmol/min/mg protein.

**Fig. 4. Northern blot analysis of rat liver PEMT mRNA.**

Five μg of poly(A)+ RNA and total RNA were separated on a 1.2% agarose/formaldehyde gel and transferred to a Hybond N* membrane (Amersham Corp.). Hybridization was performed with the cDNA probe at 42 °C in the presence of 50% formamide, and a stringent wash was performed at 60 °C in 0.1 × SSC (0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS. The oligonucleotide probes were hybridized at 42 °C in 6 × SSC, and the wash was performed at 55 °C in 6 × SSC, 0.05% sodium pyrophosphate. Panel A, RNA blot probed with random-primed, 32P-labeled, full-length PEMT cDNA. Panel B, RNA blot probed with PEMT region 328-347 5'-end-labeled oligonucleotide. Panel C, RNA blot probed with region 543-563 5'-end-labeled oligonucleotide.

**Fig. 5. Tissue distribution of PEMT protein in rat tissues.**

Rat tissues were collected immediately after total body perfusion with phosphate-buffered saline. Fifty μg of protein from the total homogenate of each tissue were separated on a 12.5% polyacrylamide, 0.1% SDS gel, transferred to nitrocellulose membranes, and probed with anti-PEMT antibody. Detection was via the enhanced chemiluminescence procedure (Amersham protocol). Tissues are labeled at the top of the figure, and molecular size markers (in kDa) at the side.

**Table IV**

Tissue distribution of PEMT activity

Tissue preparations are the same as in Fig. 5. Twenty-five mg of the total homogenate were assayed for PEMT activity with PMME as the exogenous substrate.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PEMT activity (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>10.5</td>
</tr>
<tr>
<td>Heart</td>
<td>3.9</td>
</tr>
<tr>
<td>Kidney</td>
<td>34.7</td>
</tr>
<tr>
<td>Lung</td>
<td>104.0</td>
</tr>
<tr>
<td>Muscle</td>
<td>15.0</td>
</tr>
<tr>
<td>Intestine</td>
<td>25.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>7.3</td>
</tr>
<tr>
<td>Testis</td>
<td>27.3</td>
</tr>
<tr>
<td>Liver</td>
<td>1238.0</td>
</tr>
</tbody>
</table>

**Fig. 5. Tissue distribution of PEMT protein in rat tissues.**

Rat tissues were collected immediately after total body perfusion with phosphate-buffered saline. Fifty μg of protein from the total homogenate of each tissue were separated on a 12.5% polyacrylamide, 0.1% SDS gel, transferred to nitrocellulose membranes, and probed with anti-PEMT antibody. Detection was via the enhanced chemiluminescence procedure (Amersham protocol). Tissues are labeled at the top of the figure, and molecular size markers (in kDa) at the side.
tissues was controversial (Ridgway, 1989). One proposal maintained that, as in yeast, two enzymes were involved in the conversion of PE to PC. With the purification of PEMT from rat liver microsomes in 1987 (Ridgway and Vance, 1987), the evidence clearly showed that a single enzyme was capable of catalyzing all three transmethylation reactions. Since the majority of PEMT activity was located on the ER (Vance and Vance, 1988), and there was no evidence by immunoblot analysis for more than one form of PEMT (Ridgway and Vance, 1987), the existence of a single enzyme was indicated in rat liver. The cloning and expression studies now presented unambiguously show that an antibody to the carboxyl terminus of PEMT-2 does not recognize a similar protein in the ER. Thus, PEMT-2 is substantially different from that of PEMT-1, for which at least the carboxyl-terminal sequence is consistent with this hypothesis. However, the immunoblot studies on subcellular fractions (Fig. 6B) unambiguously show that an antibody to the carboxyl terminus of PEMT-2 does not recognize a similar protein in the ER. Thus, PEMT-2 is a unique form of PEMT. Another form of PEMT (a putative PEMT-1), for which at least the carboxyl-terminal sequence is substantially different from that of PEMT-2, must be present on the ER.

The function of PEMT in liver is to convert PE to PC. Since adequate choline is usually provided to animals via the diet, PE methylation may not be essential for life. On the other hand, PEMT may have an unknown essential function, which may explain the survival of this enzyme activity during evolution. The discovery of PEMT-2 complicates the question of what is the function of PEMT. One possibility is that PEMT activity may be required in the unique membrane of liver for conversion of PE to PC. In order to target PEMT activity to that membrane, one form of PEMT (PEMT-2) may have a specific targeting protein sequence. At the moment, there is no evidence in support of this proposal.

Comparison of PEMT-2 Sequence with Other Methyltransferases—Comparison of the rat liver PEMT-2 protein sequence with that of yeast PEM2p (Fig. 2A) has revealed an overall 68% similarity of sequence with 44% identical residues. The most apparent region of homology resides from amino acids at positions 92–146 of PEMT-2. Eighty-eight percent (48/54) of the amino acids residues of this region are conserved, and 72% (39/54) are identical. Both rat liver PEMT-2 and PEM2p share common functional features with PEM1p such as methylation of PE and binding of AdoMet. The yeast PEM1p, however, catalyzes only the first methylation reaction of the three consecutive steps (i.e. PE to PMME), whereas PEM2p (Gaynor and Carman, 1990) and PEMT-2 catalyze all three transmethylations. Moreover, PEM1p is approximately 4 times as large as PEM2p and PEMT-2. Rat liver PEMT-2, PEM1p and PEM2p sequences were compared by a dot matrix method (Devereux et al., 1984), which revealed a region of highly conserved amino acid sequence (residues 92–146 in PEMT-2) (Fig. 2A). We therefore postulate that this region of PEMT-2 might be the functional center of the enzyme which would contain the substrate binding site(s).

Comparison of bacterial methyltransferases that utilize AdoMet as a substrate reveals a conserved tripeptide sequence, GXG, which may be involved in AdoMet binding (Lauster, 1989). This tripeptide motif is also present in most eucaryotic methyltransferases (Ingrosso et al., 1989). Moreover, this tripeptide motif occurs within the most conserved region of all three PEMTs (residues 92–146 in PEMT-2). Furthermore, the tripeptide sequence is located approximately 100 residues from the amino termini of the three PEMTs.
(Fig. 2A). The above results suggest that the tripeptide may be a potential binding site for S-adenosylmethionine in the PEMTs.

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