Cloning and expression of CTP:phosphoethanolamine cytidylyltransferase cDNA from rat liver

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CTP:phosphoethanolamine cytidylyltransferase (ET) is a key regulatory enzyme in the CDP-ethanolamine pathway for phosphatidylethanolamine synthesis. As a first step in the elucidation of the structure–function relationship and the regulation of ET, an ET cDNA was cloned from rat liver. The cloned cDNA encodes a protein of 404 amino acid residues with a calculated molecular mass of 45.2 kDa. The deduced amino acid sequence is very similar to that of human ET (89 % identity). Furthermore, it shows less, but significant, similarity to yeast ET as well as to other cytidylyltransferases, including rat CTP:phosphocholine cytidylyltransferase and *Bacillus subtilis* glycerol-3-phosphate cytidylyltransferase. Like human and yeast ET, rat ET has a large repetitive internal sequence in the N- and C-terminal halves of the protein. Both parts of the repeat contain the HXGH motif, the most conserved region in the N-terminal active domain of other cytidylyltransferases, indicating the existence of two catalytic domains in ET. The hydropathy profile revealed that rat ET is largely hydrophilic and lacks a hydrophobic stretch long enough to span a bilayer membrane. There was no prediction for an amphipathic α-helix. Transfection of COS cells with the cDNA clone resulted in an 11-fold increase in ET activity, corresponding to an increase in the amount of ET protein as detected on a Western blot. Determination of the ET activity during liver development showed a 2.5-fold increase between day 17 of gestation and birth (day 22) and the amount of ET protein changed accordingly. Northern blot analysis showed that this was accompanied by an increase in the amount of ET mRNA. Between day 17 of gestation and birth, the amount of mRNA in fetal rat liver increased approx. 6-fold, suggesting the regulation of ET at both pretranslational and post-translational levels during rat liver development.

Key words: gene expression, liver development, phosphatidylethanolamine.

INTRODUCTION

Phosphatidylethanolamine (PtdEtn) is a major constituent of cellular membranes in both eukaryotes and prokaryotes [1]. This phospholipid can be synthesized by base exchange with preexisting phospholipids, by the decarboxylation of phosphatidylserine or by the incorporation of ethanolamine (Etn) via the CDP-ethanolamine (CDP-Etn) pathway (reviewed in [2]). Studies *in vivo* with labelled substrates [3] and experiments with freshly isolated hepatocytes [4] have shown that, in liver, the CDP-Etn pathway is the principal route in the synthesis of PtdEtn *de novo*. Regulation of PtdEtn synthesis can take place at multiple sites in the CDP-Etn pathway, but CTP:phosphoethanolamine cytidylyltransferase (ET; EC 2.7.7.14) catalysing the synthesis of CDP-Etn from phosphoethanolamine is considered to be the most important regulatory enzyme of the pathway [2].

In 1975, ET was purified 1000-fold from a post-microsomal supernatant from rat liver [5]. More recently, Vermeulen et al. [6] developed a new procedure to purify ET to apparent homogeneity from rat liver. This procedure yielded a protein with a molecular mass of 49.6 kDa under both reductive and denaturing polyacrylamide gel conditions. Gel-filtration experiments revealed that the native enzyme occurs as a dimer [7].

Despite the purification and preliminary characterization of the protein, no information is available about the amino acid sequence of rat liver ET or its coding nucleotide sequence. Only very recently was the *ECT1* gene coding for ET of the yeast *Saccharomyces cerevisiae* isolated [8]; half a year later the cloning of a human cDNA for ET was reported [9]. The predicted amino acid sequence of yeast and human ET showed a high degree of similarity (36 % identity), which extends across the entire length of both proteins. Interestingly, both proteins contain a large repetitive sequence in their N- and C-terminal halves, which might imply the presence of two catalytic domains in human and yeast ET [9].

The most similar regions in the repetitive sequence are also similar to some well-conserved regions in the N-terminal part of members of the cytidylyltransferase superfamily, including rat and yeast CTP:phosphocholine cytidylyltransferase (CT) and glycerol-3-phosphate cytidylyltransferase (GCT) of *Bacillus subtilis* [10]. On the basis of the high degree of similarity, it has been proposed that the N-terminal region contains the catalytic domain of these cytidylyltransferases.

The properties of CT, the key regulatory enzyme in the CDP-choline pathway for phosphatidylcholine synthesis, have been studied extensively [11]. Several mechanisms, such as (1) phosphorylation–dephosphorylation [12–14], (2) reversible translocation [11,15] and (3) regulation of the enzyme at a pretranslational level [16–18], have a role in the regulation of CT activity. The sequences of cDNA coding for CT of several species, including rat [19], human [20] and *Arabidopsis thaliana* [21], have been reported. In addition, the genes coding for CT of

Abbreviations used: CDP-Etn, CDP-ethanolamine; CT, CTP:phosphocholine cytidylyltransferase; ER, endoplasmic reticulum; ET, CTP:phosphoethanolamine cytidylyltransferase; Etn, ethanolamine; GCT, glycerol-3-phosphate cytidylyltransferase; ORF, open reading frame; PtdEtn, phosphatidylethanolamine; RACE, rapid amplification of cDNA ends.

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the yeast *S. cerevisiae* [22] and the malaria parasite *Plasmodium falciparum* [23] have been determined. CT is thought to contain a globular N-terminal catalytic domain, well conserved between different organisms [21,24].

To elucidate the relationship between the structure and the function of ET we report the molecular cloning of a cDNA coding for rat liver ET. We used this ET cDNA for the transfection of COS-7 cells. Furthermore, the ET cDNA was used as a probe in subsequent hybridization experiments to gain some insight into the regulation of ET at the level of mRNA synthesis during the development of rat liver.

EXPERIMENTAL

Materials and animals

All degenerate oligonucleotides and ET-specific primers used in this study were synthesized by Pharmacia Biotech Benelux (Roosendaal, The Netherlands). Oligo(dT)-anchor primer and PCR anchor primer were provided with the 5′/3′ rapid amplification of cDNA ends (RACE) kit, which was from Boehringer Mannheim (Mannheim, Germany). RNAzol™ B was purchased from Tel-Test; oligo(dT)-cellulose columns were from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.). A rat liver cDNA library, from which the cDNA coding for CT had also been cloned, was a gift from R. Cornell [19]. *Taq* DNA polymerase, Mg^{2+}-free thermophilic DNA Poly Reaction buffer and $MgCl_2$ were from Promega (Madison, WI, U.S.A.). Expand™ High Fidelity PCR system was obtained from Boehringer Mannheim. Geneclean Kit was purchased from BIO 101 (Vista, CA, U.S.A.). pMOS*Blue* T-vector kit and pMOS*Blue* competent cells were from Amersham Life Science (Little Chalfont, Bucks., U.K.). Wizard® Plus Minipreps DNA purification system was obtained from Promega, and Qiagen Plasmid Maxi Kit was from Qiagen GmbH (Hilden, Germany). [α-^{32}P]dATP (3000 Ci/mmol) and [2-^{14}C]ethanolamine hydrochloride (57 mCi/mmol) were purchased from Amersham International. Phospho[2-^{14}C]ethanolamine was prepared enzymically from [2-^{14}C]ethanolamine with partly purified ethanolamine kinase, as described previously [7]. COS-7 (ATCC CRL-1651) cells and plasmid PSCT-ΔHP were kindly provided by P. E. Holthuizen (Department of Physiological Chemistry, Utrecht University, The Netherlands). Immobilon-P was from Millipore (Bedford, MA, U.S.A.) and Nytran N from Schleicher and Schuell (Dassel, Germany). Salmon sperm DNA was from Sigma (St. Louis, MO, U.S.A.). All other chemicals were from Pharmacia Biotech, Gibco BRL or Merck (Darmstadt, Germany).

Male (200–250 g) and timed pregnant female Wistar rats (HsdCpb:Wu, Harlan-CPB) were obtained from our animal facilities. The animals had free access to water and a standard commercial chow pellet diet.

Protein purification and internal sequence analysis

ET was purified from rat liver exactly as described by Vermeulen et al. [6]. Approx. 40 μg of purified protein was precipitated with trichloroacetic acid [1 % (w/v) final concentration] and resolved by SDS/PAGE, which was performed with a 12 % (w/v) polyacrylamide gel. After the gel had been stained with 0.1 % Coomassie Brilliant Blue, the band containing ET was excised. Subsequently, ET was digested *in situ* with trypsin and the resulting peptides were extracted and purified by reverse-phase HPLC. Suitable fragments were selected and sequenced by Edman degradation with an automated sequenator (Model 477A; Applied Biosystems) by Eurosequence (Groningen, The Netherlands). The sequences of the three peptides analysed are underlined in Figure 1(B).

Oligonucleotides

Degenerate oligonucleotides were used as primers in PCR with cDNA from a rat liver cDNA library as the template. ET 1, 5′-GTGTAAGCCCGCGATIACGTAGG-3′, was constructed on the basis of amino acid sequence PYVIAGLH of tryptic peptide 2 and the nucleotide sequence of the cDNA region coding for the same peptide in human ET (GenBank/EBI Data Bank accession number D84307 [9]) and was complementary to the sense strand. Universal primer UP, 5′-GTAAAACGACGGCCAGT-3′, complementary to the sequence of vector pUC19, was used as the sense primer.

ET-specific primers were used in 5′/3′ RACE–PCR in which cDNA synthesized from rat liver poly(A)$^+$ RNA was used as the template. The anti-sense primers ET 2 (5′-GCACCAGCCA-CATAGATGACTG-3′), ET 3 (5′-GGATGTCTGTAGGAAC-TGGGAC-3′) and ET 4 (5′-GCACTGGGAGGAGACTTC-TGAG-3′), used in the 5′ RACE–PCR, and the sense primers ET 5 (5′-CCATAGCAGCCAGGAGATGTCC-3′) and ET 6 (5′-CAGTCATCTATGTGGCTGGTGC-3′), used in the 3′ RACE–PCR, were all deduced from the sequence of the cDNA region between primers ET 1 and UP. Oligo(dT)-anchor primer (5′-GACCACGCGTATCGATGTCGAC$(T)_{16}$V-3′) and PCR anchor primer (5′-GACCACGCGTATCGATGTCGAC-3′), provided in the 5′/3′ RACE kit, were used as 5′ primer in the 5′ RACE–PCR and as 3′ primer in the 3′ RACE–PCR. ET-specific primers ET 7 (5′-TGTCGCGGGAGCTGCCAGGACTT-3′) and ET 8 (5′-GGAAAGGACTGAGAGCCTGCTGCA-3′), used to amplify the complete ET cDNA, were deduced from the sequences of the cDNA fragments constructed in the 5′/3′ RACE–PCR.

RNA isolation and cDNA synthesis

Total RNA was isolated from rat liver by the RNAzol B method. Poly(A)$^+$ RNA (mRNA) was isolated by affinity chromatography on oligo(dT)cellulose and used as a template for the synthesis of cDNA. To generate cDNA, oligo(dT)-anchor or ET 2 primers were used with the reagents provided with the 5′/3′ RACE kit in accordance with the manufacturer's instructions.

PCR and RACE–PCR

An ET cDNA fragment was amplified from a cDNA library between primers ET 1 and UP. The PCR reaction mixture (50 μl) contained 200 ng of template cDNA, 15 pmol of ET 1, 15 pmol of UP primer, 1 unit of *Taq* polymerase, 0.4 mM of each deoxynucleoside triphosphate (dNTP) and 2.5 mM $MgCl_2$ in a PCR buffer consisting of 10 mM Tris/HCl (pH 9.0 at 25 °C), 50 mM KCl and 0.1 % (v/v) Triton X-100. The cDNA was denatured for 1 min at 93 °C and amplified in 35 cycles of denaturation at 93 °C for 1 min, annealing at 53 °C for 1 min and extension at 72 °C for 1 min.

To amplify ET sequences at the 5′ and 3′ ends of mRNA, cDNA was synthesized (see the section on RNA isolation and cDNA synthesis) and used as a template for PCR. The reaction mixture for 5′ RACE–PCR (50 μl) contained 5 μl of dA-tailed cDNA synthesized from mRNA with primer ET 2, 12.5 pmol of ET 3, 12.5 pmol of oligo(dT)-anchor primer, 0.2 mM dNTP, 2.5 units of *Taq* polymerase and 1.5 mM $MgCl_2$ in the above-mentioned PCR buffer. PCR conditions were: denaturation at 94 °C for 2 min, 10 cycles of 94 °C for 15 s, 55 °C for 30 s,

and 72 °C for 40 s and 25 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 40 s plus a cycle elongation time of 20 s for each cycle. A nested PCR was performed with primers ET 4 and PCR anchor. For this reason, 1 μl of first-round PCR product, diluted 1:20, was used as the template and PCR conditions were as described above except for the annealing temperature, which was 62 °C. For 3′ RACE–PCR two rounds of PCR were also performed. cDNA was generated from mRNA with oligo(dT)-anchor primer and a 1 μl aliquot was used as template in a PCR with primers ET 5 and PCR anchor. Cycling conditions were the same as those for the 5′ RACE–PCR, but with an annealing temperature of 62 °C. The PCR product was diluted 1:20 and 1 μl was used in the second round of PCR. The nested PCR was performed with primers ET 6 and PCR anchor as described above but with an annealing temperature of 55 °C.

Construction and amplification of the cDNA region coding for the entire ET protein was achieved by PCR with primers ET 7 and ET 8 on cDNA synthesized for the 3′ RACE–PCR (see above) as the template. For this PCR, the Expand™ High Fidelity PCR system with the corresponding enzyme mix (2.6 units) and buffer containing 1.5 mM $MgCl_2$ was used. PCR conditions were as described for 5′/3′ RACE–PCR except for annealing (60 °C for 30 s) and elongation (68 °C for 1 min).

Cloning

PCR products were purified by agarose-gel electrophoresis and extracted from the gel by the glassmilk method (Geneclean). The purified cDNA was ligated into the pMOS*Blue* vector and transformed into MOS*Blue* competent cells by heat shock at 42 °C, in accordance with the manufacturer's instructions. The MOS*Blue* competent cells and the MOS*Blue* vector were used for the maintenance and amplification of all PCR products mentioned. The bacterial cells were grown in Luria–Bertani medium supplemented with 100 μg/ml ampicillin.

DNA sequencing and sequence analysis

To isolate plasmid DNA from MOS*Blue* competent cells, Wizard Plus Miniprep or Qiagen plasmid maxi kit was used. After annealing of fluorescent Cy5-labelled T7 promotor primer and U-19-mer primer, complementary to vector sequences on both sides of the *Eco*RV cloning site, the sequence of the inserted cDNA was determined by the dideoxy chain termintion method [25] with an Automated Laser Fluorescent DNA Sequencer (Pharmacia). The PCR product spanning the complete ET coding region was sequenced on both strands by Eurogentec (Seraing, Belgium).

Nucleotide and deduced amino acid sequences were compared with sequences in the non-redundant databases available at the National Center for Biotechnology Information (NCBI) by using the BLAST program. Sequence analysis and alignments were performed with the CGC program package from Wisconsin University. The Prosite database (EBI server) was used for sequence similarity searches to find putative consensus motifs for phosphorylation sites or lipid-binding domains in ET. Hydrophobicity was analysed by the method of Kyte and Doolittle [26] and α-helix amphiphilicity by the algorithm of Chou and Fasman [27].

Transfection of COS-7 cells with ET cDNA and assay of ET activity

A cDNA fragment harbouring the region coding for the entire ET protein was excised from the pMOS*Blue* vector with *Bam*HI and *Pst*I. The resulting 1.4 kb fragment was ligated between the *Bam*HI and *Pst*I sites of the expression vector PSCT-ΔHP. This vector, which is a *Hin*dIII–*Pvu*II deletion derivative of PSCT Gal X-556 [28], contains a simian virus 40 origin of replication for amplification to high copy number in COS monkey cells and a human cytomegalovirus promotor for the transcription of inserted cDNA. Plasmid DNA was amplified by transformation to JM109 cells and isolated with Qiagen plasmid maxi kit. COS-7 cells (5×10^5) were grown on 20 cm^2 dishes for 24 h before transfection with 10 μg of isolated PSCT-ΔHP containing the ET cDNA insert (PSCTΔHP-ET). Control cells were transfected with empty plasmid. Transfection was performed by precipitation with calcium phosphate in Hepes-buffered saline [2 × HBS: 40 mM Hepes/274 mM NaCl/10 mM KCl/1.4 mM $Na_2HPO_4 \cdot 2H_2O$ (pH 7.15)]. At 48 and 72 h after transfection, cells were lysed in 300 μl of ice-cold lysis buffer [25 mM Tris/phosphate (pH 7.8)/15 % (v/v) glycerol/1 % (v/v) Triton X-100/8 mM $MgCl_2$/1 mM dithiothreitol]. After lysis for 10 min at 4 °C, cells were scraped off the dishes and the resulting cell homogenate was assayed for ET activity. The activity of ET was measured in 40 μl cell homogenate (approx. 160 μg of protein) as described by Tijburg et al. [7], except that phospho[2-^{14}C]ethanolamine and CDP[2-^{14}C]ethanolamine were separated on silica-gel H TLC plates with 96 % (v/v) ethanol/0.5 % NaCl/25 % (v/v) NH_4OH (50:50:5, by vol.) as the developing solvent.

Immunoblotting

Cell homogenate protein (45 μg) from transfected COS cells or rat liver cytosol (50 μg) was resolved by SDS/PAGE [12 % (w/v) gel] and transferred to Immobilon-P PVDF membranes by electrophoretic blotting [29]. Membranes probed with anti-ET antiserum [6] were revealed with an enhanced chemiluminescence development reagent (ECL™ system; Amersham) in accordance with the manufacturer's instructions.

Northern blot analysis

mRNA from prenatal (days 17, 18, 19, 20, 21 and 22 of gestation), post-natal (immediately after birth, days 1 and 2 post-natal) and adult rat liver was isolated as described in the section on RNA isolation and cDNA synthesis. For each time point, four pregnant rats were killed and of each rat the livers of all fetuses were pooled. mRNA was fractionated by electrophoresis on a 1.2 % (w/v) agarose gel containing formaldehyde, then transferred to Nytran N by capillary blotting with 20 × SSC (3.0 M NaCl/0.3 M sodium citrate). The membrane was prehybridized at 42 °C for 5 h in a prehybridization buffer containing 6 × SSPE (20 × SSPE = 3.0 M NaCl/0.2 M sodium phosphate/0.02 M EDTA), 10 × Denhardt's solution (5 × Denhardt's = 0.1 % Ficoll/0.1 % polyvinylpyrrolidone/0.1 % BSA), 1 % (w/v) SDS and 100 μg/ml salmon sperm single-stranded DNA. After addition of the 1.1 kb 3′ RACE second-round PCR product as the cDNA probe, ^{32}P-labelled by random priming, the blot was hybridized overnight at 42 °C. The hybridization solution consisted of 6 × SSPE, 5 × DH, 1 % SDS, 50 % (v/v) formamide, 100 μg/ml salmon sperm DNA and 10 % (w/v) dextran sulphate. The membrane was then washed twice with 2 × SSPE/0.3 % SDS at room temperature for 10 min, and subsequently with 0.5 × SSPE/0.2 % SDS at 42 °C for 20 min, 0.2 × SSPE/0.2 % SDS at 50 °C for 30 min, 0.1 × SSPE/0.1 % SDS at 50 °C for 30 min, 0.1 × SSPE/0.1 % SDS at 60 °C for 30 min and 0.05 × SSPE/0.1 % SDS at 60 °C for 20 min. After exposure to X-ray film and quantification of the signals by phosphorimaging, the blot was stripped and reprobed with ^{32}P-labelled β-actin cDNA (clone pHFβA-3′UT-HF, 0.4 kb

insert encoding human cytoplasmic β-actin). The washing procedure as described for ET was used with $0.2 \times$ SSPE/0.2 % SDS at 50 °C for 30 min as final wash step.

RESULTS

Strategy for cloning of ET cDNA

In an attempt to obtain oligonucleotides that could be used as primers for the amplification of ET cDNA from a rat liver cDNA library by PCR, we first determined the amino acid sequence of peptides of purified rat liver ET protein. Because the N-terminus of ET was blocked, three internal tryptic peptides were sequenced (underlined in Figure 1B). Peptides 1 and 3 showed 100 % identity with peptides corresponding to residues 47–64 and 280–290 respectively in human ET, which had been cloned during the course of our study. Peptide 2 was more than 90 % identical with human ET residues 243–256. In contrast with peptides 2 and 3, peptide 1 also showed significant similarity to a peptide in yeast ET (residues 41–51). Because this high degree of similarity to ET, especially to mammalian ET, confirmed that peptides 1, 2 and 3 were from rat ET, degenerate oligonucleotides were deduced for use in a PCR (Figure 1A).

A cDNA sequence specifically coding for part of rat ET was derived by amplification from a rat liver cDNA library. When degenerate oligonucleotides derived from peptide 1 were used, no amplification product could be obtained. However, PCR amplification with degenerate oligonucleotides encoding peptide 2 (primer ET 1) and the non-specific primer UP, complementary to a sequence of vector pUC19, resulted in a PCR product of approx. 600 bp. This PCR product was cloned; sequencing showed that the cDNA fragment coded for a peptide of 191 residues that was very similar to other cytidylyltransferases such as human and yeast ET, CT from various species and *B. subtilis* GCT. Of the 191 residues of the rat sequence, 165 were identical with the sequence comprising residues 78–250 in human ET (Figure 2A). From the 600 bp PCR product, ET-specific primers were deduced that were used in specific PCRs performed to determine the 5′ and 3′ parts of the ET cDNA sequence.

The sequences of the 5′ and 3′ halves of rat ET cDNA were obtained in two separate RACE–PCRs. Because an intact full-length cDNA, particularly the 5′ end of the cDNA, is rarely recovered from cDNA libraries, cDNA made from rat liver mRNA was used as the template in the 5′ and 3′ RACE–PCRs.

In the 5′ RACE–PCR, cDNA between primers ET 3 and oligo(dT)-anchor was amplified. The resulting PCR product was subjected to a second round of PCR with primers ET 4 and PCR anchor. This yielded a PCR product of approx. 680 bp, which was cloned and sequenced. This 5′ RACE–PCR product encoded a peptide of 214 residues in which the sequence of tryptic peptide 1 also could be found (Figure 1A). The amino acid sequence was 80 % similar to the amino acid sequence of human ET. Close to the 5′ end of the PCR product, a sequence coding for the amino acids MIRNG was found (Figure 1B), corresponding to the first five residues of human ET. This confirmed that the 5′ RACE–PCR product represented the 5′ terminus of rat ET, assuming identical start sequences for rat and human ETs.

In the 3′ RACE–PCR, rat ET cDNA was also amplified in two rounds of PCR. In the first round of PCR, ET 5 and PCR anchor were used; in the nested PCR, ET 6 and PCR anchor were used. This resulted in an abundant 1100 bp product; sequencing showed that the cDNA fragment encompassed the regions coding for tryptic peptides 2 and 3 (Figure 1A). As with the 5′ RACE–PCR product, the amino acid sequence of the 3′ RACE–PCR product also presented a high degree of similarity (89 %) to human ET. The nucleotide sequence of the 3′ RACE–PCR product [from primer ET 6 to the poly(A) tail] exhibited 69 % similarity to the same region in human ET cDNA.

From the 5′ and 3′ RACE–PCR products, ET-specific primers ET 7 and ET 8 were derived, which were used as 5′ primer and 3′ primer respectively in a PCR performed to generate the full-length ET cDNA. A PCR product of 1425 bp was obtained, which was of the expected size as calculated from the combined sequences of the 5′ and 3′ RACE–PCR products. The 1425 bp product was cloned into pMOS*Blue* and transformed to competent cells. Of all the transformants that grew as white colonies on ampicillin–tetracycline agar plates impregnated with isopropyl β-D-thiogalactoside and 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-Gal'), one representative clone was selected for a determination of the complete ET coding cDNA sequence. The cDNA sequence and the deduced amino acid sequence are shown in Figure 1(B). A map of all the primers used in the sequencing strategy is shown in Figure 1(A).

Features of the nucleotide and deduced amino acid sequences of rat ET

The nucleotide sequence of the cloned cDNA was determined on both strands and consisted of a total of 1846 bp (Figure 1B). It contained a 1215 bp open reading frame (ORF) starting from base 45 with an ATG codon and ending at base 1259 (Figure 1B). This ATG codon at position 45 is very probably the start codon for translation, because it is in the consensus sequence for translation initiation [30], (A/G)XXATG(A/G). This view is supported by a sequence comparison with human ET, because the start sequence MIRNG encoded by the rat cDNA sequence is the same as that encoded by human ET cDNA (Figure 2A).

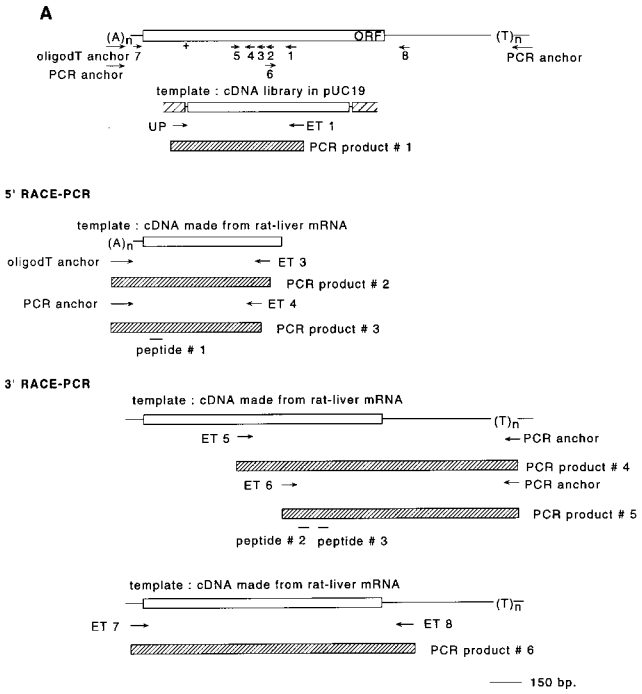
The complete ORF codes for a protein of 404 residues with a calculated molecular mass of 45.2 kDa. This predicted molecular mass corresponds well with the molecular mass of 49.6 kDa estimated by SDS/PAGE of purified rat ET protein [6]. As with human and yeast ET, rat ET has a large internal repetitive sequence (Figure 2B). Both sequences of the repeat contain the HXGH motif (residues 35–38 and 244–247), which is also present in the most conserved N-terminal region of members of the cytidylyltransferase superfamily.

Analysis of the hydrophobicity profile revealed that the overall sequence of ET does not contain a hydrophobic region long enough to form a transmembrane domain (Figure 3A). The longest unbroken stretches of hydrophobic residues are not more than four residues long and are present in the N-terminal area (residues 51–54) and in the central domain (residues 153–156 and 233–236). Secondary structure analysis with the Chou–Fasman algorithm did not predict any region that could form an amphipathic α-helix (Figure 3B).

When putative consensus motifs were searched for in the Prosite database, three potential myristoylation sites were found at the N-terminus of ET (GAGGAA at position 7–12, GGAAGL at position 9–14 and GGQRTV at position 18–23). However, no glycine residue is present at position 2, which is required for the covalent attachment of a myristoyl chain [31]. The Prosite database was also used to find consensus sequences for phosphorylation sites. One possible phosphorylation site for protein kinase C (TVR at position 22) and one for casein kinase 2 (DTEE at position 58) were suggested.

Sequence similarity to other cytidylyltransferases

The overall sequence of the predicted rat ET protein shows approx. 25 % similarity to other cytidylyltransferases, including



B

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1     TGTGTCGCGGGAGCTGCCAGGACTTGCGGGGGGCCTCTGGTGCCATGATCCGGAACGGG
1                                                 M  I  R  N  G
60    CACGGGGCTGGCGGCGCTGCTGGGCTGAAGGGTCCGGGCGGCCAGCGCACCGTGCGGGTG
6     H  G  A  G  G  A  A  G  L  K  G  P  G  G  Q  R  T  V  R  V
120   TGGTGCGATGGCTGCTATGACATGGTGCATTACGGCCACTCCAACCAGCTACGCCAGGCG
26    W  C  D  G  C  Y  D  M  V  H  Y  G  H  S  N  Q  L  R  Q  A
180   CGGGCCATGGGGGACTACCTCATTGTGGGCGTGCATACTGACGAGGAGATTGCCAAGCAT
46    R  A  M  G  D  Y  L  I  V  G  V  H  T  D  E  E  I  A  K  H
240   AAGGGGCCCCCGGTGTTTACCCAGGAGGAGCGGTACAAGATGGTACAGGCCATCAAGTGG
66    K  G  P  P  V  F  T  Q  E  E  R  Y  K  M  V  Q  A  I  K  W
300   GTGGATGAAGTGGTACCGGCTGCTCCCTACGTCACCACACTGGAGACACTGGACAAGCAC
86    V  D  E  V  V  P  A  A  P  Y  V  T  T  L  E  T  L  D  K  H
360   AACTGTGACTTCTGCGTTCATGGCAATGACATCACGCTGACAGTAGATGGCCGAGATACC
106   N  C  D  F  C  V  H  G  N  D  I  T  L  T  V  D  G  R  D  T
420   TACGAGGAAGTGAAGCAGGCTGGGAGGTACAGAGAGTGCAAACGCACCCAGGGTGTGTCC
126   Y  E  E  V  K  Q  A  G  R  Y  R  E  C  K  R  T  Q  G  V  S
480   ACCACAGACCTCGTGGGTCGCATGCTGCTGGTGACCAAGGCCCACCATAGCAGCCAGGAG
146   T  T  D  L  V  G  R  M  L  L  V  T  K  A  H  H  S  S  Q  E
540   ATGTCCTCTGAGTACCGGGAATATGCTGACAGTTTGGCAAGCCCCCTCACCCGACACCT
166   M  S  S  E  Y  R  E  Y  A  D  S  F  G  K  P  P  H  P  T  P
600   GCCGGGGACACACTTTCCTCAGAAGTCTCCTCCCAGTGCCCTGGGGGCAGAGCCCCTGG
186   A  G  D  T  L  S  S  E  V  S  S  Q  C  P  G  G  Q  S  P  W
660   ACAGGGGTGTCCCAGTTCCTACAGACATCCCAGAAGATCATCCAGTTTGCTTCTGGGAAG
206   T  G  V  S  Q  F  L  Q  T  S  Q  K  I  I  Q  F  A  S  G  K
720   GAGCCCCAGCCCGGGGAGACAGTCATCTATGTGGCTGGTGCCTTTGACCTGTTCCACATC
226   E  P  Q  P  G  E  T  V  I  Y  V  A  G  A  F  D  L  F  H  I
780   GGGCACGTGGACTTCCTACAGGAGGTGCACAAGCTAGCCAAGAGGCCCTACGTCATCGCC
246   G  H  V  D  F  L  Q  E  V  H  K  L  A  K  R  P  Y  V  I  A
840   GGCCTACACTTTGACCAGGAAGTAAACCGGTACAAGGGCAAGAACTACCCCATCATGAAC
266   G  L  H  F  D  Q  E  V  N  R  Y  K  G  K  N  Y  P  I  M  N
900   CTGCATGAGCGGACTCTCAGTGTGCTGGCCTGCCGGTATGTTTCAGAAGTGGTGATTGGG
286   L  H  E  R  T  L  S  V  L  A  C  R  Y  V  S  E  V  V  I  G
960   GCACCGTACTCCGTCACAGCAGAGCTCCTGAATCATTTCAAGGTGGACCTGGTGTGTCAT
306   A  P  Y  S  V  T  A  E  L  L  N  H  F  K  V  D  L  V  C  H
1020  GGGAAGACAGAAATTGTACCCGACAGGGATGGGTCTGACCCCTACGAGGAGCCCAAGAGA
326   G  K  T  E  I  V  P  D  R  D  G  S  D  P  Y  E  E  P  K  R
1080  AGAGGCATTTTCTGTCAGATTGACAGTGGCAGTGACCTCACTACAGACCTGATTGTGCAA
346   R  G  I  F  C  Q  I  D  S  G  S  D  L  T  T  D  L  I  V  Q
1140  AGGATCATCAAGAACAGGTTGGAGTACGAAGCACGGAATCAGAAGAAGGAAGCCAAGGAA
366   R  I  I  K  N  R  L  E  Y  E  A  R  N  Q  K  K  E  A  K  E
1200  TTAGCCTTTTTGGAGGCCCTGAGGCAGCAGGAGGCACAGCCCAGAGGGGAGACTGACTAG
386   L  A  F  L  E  A  L  R  Q  Q  E  A  Q  P  R  G  E  T  D  *
1260  CTTCTGACCCGGAGACGTCATTCACGCCGTTGCCCTGTGCCCACCTCATCTCTCCCTGCC
1320  CTGCTCTGCTTCTGTGTCTTGGTGTCAGCTCACACAATTCCAAAGGAAACTGGCCTTGCT
1380  GAGGGTGCTGTCCCTGCCTGCCTGCAGCAGGCTCTCAGTCCTTTCCACTGAGCTGCTCAG
1440  AGAGGGTAGCTAGCCCAGTGGGGCAGCCCCGTGGACAGGATAAGCAGACGGCACCTGTGA
1500  CTGGAGGATTGTTGCTGTGTATCCTTGGCATCCACGGGTTCCAACTGCCCACTGTCCCAG
1560  TTTGGCTGGGGATACCCCTCCTGCCTGACTGGAAGCTGCACTTCTGCCCACCGAGCTTTG
1620  GTGAAAGGTACAGAGGCTTTGGGGACCTCTGTTCCTGGGCCACCCTGCACGTGGGCACCC
1680  TCTACCTTGGGGCACGTTCTAGCACCCCATTCCTGACTCCTAGAAGATGCACTTGCCCCG
1740  GGTGGCTGGGCCAGCGTGGCTGCCCTCCGCACAGACTTCCTGGTCCTCGTTTGTACCTCA
1800  GTGGCTGATGTAAACCTCTTTGGCTCAACTGAATAAAGCCTAGTGGG
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Figure 1 Nucleotide and deduced amino acid sequences of rat ET cDNA

(**A**) Sequencing strategy. The open boxes represent (parts of) the ORF. PCR products are depicted by solid bars and hatched bars represent pUC19 sequences. The arrows indicate the position and direction of the primers used in PCRs. The positions of the tryptic peptides of purified rat ET protein are shown. The plus sign denotes the position of the farthest upstream base pair present in the cDNA library. (**B**) The nucleotide sequence of rat ET cDNA and the predicted amino acid sequence of the translation product (single-letter code). Residue numbers for nucleotides and amino acids are shown at the left. The asterisk denotes the termination codon. The sequences of the three tryptic peptides are underlined.

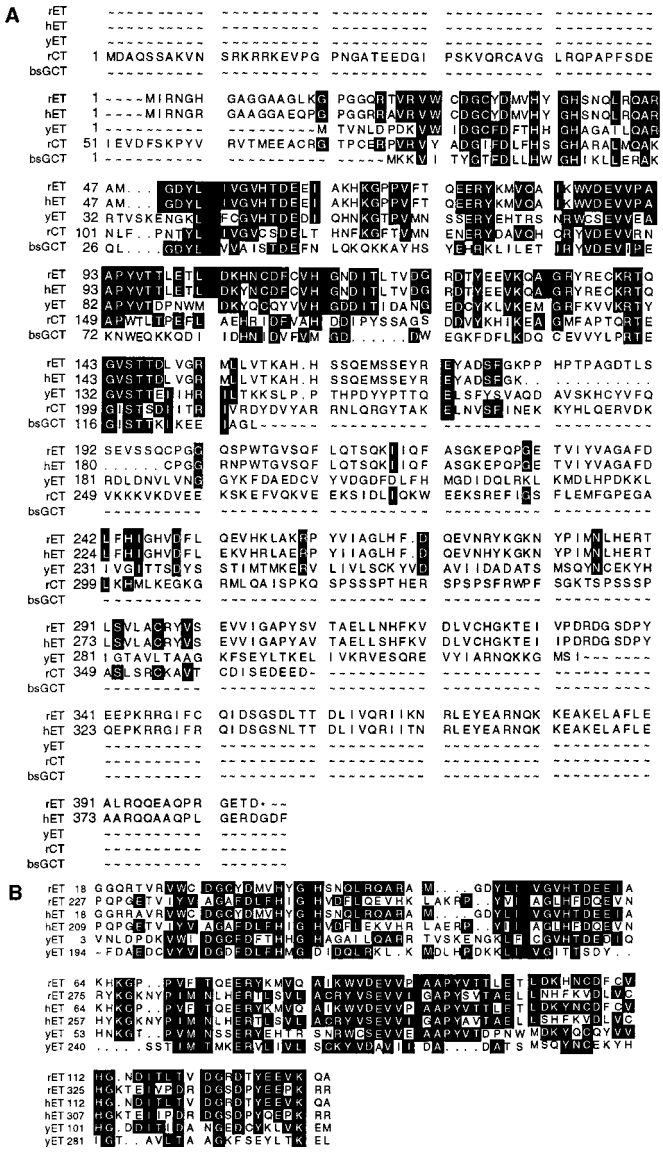

Figure 2 Alignment of amino acid sequences of rat ET and other cytidylyltransferases

(**A**) The amino acid sequences of rat ET (rET), human ET (hET; accession number D84307), yeast ET (yET; D50644), rat CT (rCT; L13245) and *B. subtilis* GCT (bsGCT; M57497) are compared. (**B**) Sequence alignment of the N-terminal and C-terminal halves of the internal repetitive sequence of rat ET (rET), human ET (hET) and yeast ET (yET). Alignments were made with the program PILEUP of the CGC program package of Wisconsin University. Residues that are identical in three or more sequences are shaded in black. Gaps in alignments are indicated by dots. Residue numbers of amino acids are shown at the left.

human and yeast ET, rat CT and *B. subtilis* GCT (Figure 2A). When compared with human ET only, the similarity is as high as 89%. Rat ET (404 residues) is somewhat longer than yeast ET (323 residues), in both the N-terminal and the C-terminal regions.

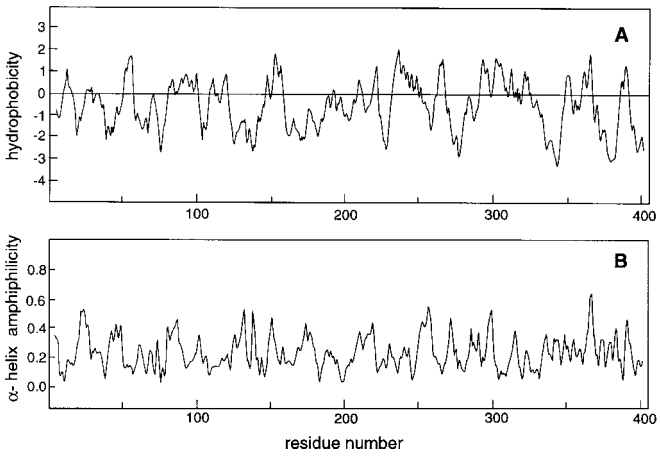

Figure 3 Hydropathy and α-helix amphiphilicity profiles of the predicted protein sequence of rat ET

(**A**) Hydropathy profile. Hydrophobicity was analysed by the method of Kyte and Doolittle [26]. Positive values represent hydrophobicity. (**B**) α-Helix amphiphilicity profile. The sequence was analysed for an amphipathic α-helix with the algorithm of Chou and Fasman [27]. The average hydropathy value (**A**) or amphiphilicity value (**B**) in a frame of seven residues is plotted against the residue number of the central amino acid.

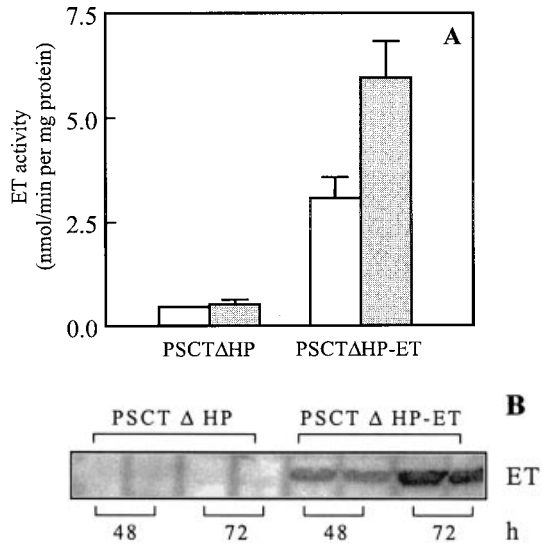

Figure 4 ET activity and protein content in transfected COS cells

COS-7 cells were transfected with the expression vector PSCT-ΔHP containing a 1.4 kb ET cDNA insert (PSCTΔHP-ET) or with an empty plasmid (PSCTΔHP). (**A**) At 48 h (open bars) and 72 h (grey bars) after transfection, cells were disrupted and assayed for ET activity. Values are means ± S.D. for duplicate transfections, each assayed in duplicate. (**B**) The amount of ET protein in total cell homogenate was determined in duplicate by immunoblotting.

It is more or less the same size as human ET (389 residues); however, compared with human ET, rat ET has an extra 18-residue insertion in the central domain (residues 180–197). When this insertion is not taken into account, 93% of the remaining residues are identical with those in the sequence of human ET. The similarity between rat, human and yeast ET extends across the entire lengths of the proteins but they share the greatest similarity in the part comprising rat ET residues 23–251 and weaker similarity in the N-terminal and C-terminal regions. The central part contains a large repetitive sequence in the N-terminal and C-terminal halves.

Alignment of these N-terminal and C-terminal halves revealed a small but significant similarity of 33% in rat and human and 23% in yeast ET (Figure 2B). Sequence similarity between the ET internal repeat and other cytidylyltransferases was limited to their N-terminal halves. Further upstream of this region, sequences displayed only marginal similarity to each other (results not shown). Both halves of the internal repetitive sequence of ET and also the N-terminal domain of the other cytidylyltransferases contain the HXGH motif. In addition, other conserved residues presumed to be very important for catalytic activity in cytidylyltransferases, such as Asp-32 and the sequence RTQGVSTT (residues 140–147), are present in rat ET.

Rat ET resembled the other cytidylyltransferases in that they lack an α-helical domain or a hydrophobic region long enough to function as a membrane-spanning segment. The rat ET sequence contains 31% hydrophobic amino acids; this is comparable to the percentage in other cytidylyltransferases such as rat CT (30%) and yeast ET (37%).

Expression of ET in COS cells

After transfection of COS-7 cells with ET cDNA inserted in plasmid PSCT-ΔHP, ET activity in cell homogenates was induced 6.4-fold (at 48 h) and 11.4-fold (at 72 h) in comparison with ET activity in COS cells transfected with empty vector (Figure 4A). Immunoblotting revealed that the stimulation of ET activity corresponded to an increase in the amount of ET protein (Figure 4B) in the cell homogenates of transfected cells.

ET activity, amount of protein and mRNA level during rat liver development

We studied the effect of liver growth on the activity and expression of ET, by using the developing liver as a model system. Figure 5(C) shows that there is a gradual increase in ET activity between day 17 of gestation and birth (day 22). From then onwards, ET activity stayed constant until the adult state. The amount of ET protein (Figure 5B), as determined by immunoblotting with an ET-specific antibody [6], showed a similar profile to that of ET activity.

To determine whether ET activity and protein are regulated at a pretranslational level in developing liver, we performed Northern blot analyses. With the 1.1 kb 3′ RACE–PCR product as a probe for hybridization with adult rat liver mRNA on a Northern blot, we detected a major transcript of approx. 2 kb (Figure 5A). The size of the transcript reflects the ORF plus the 3′ non-coding region as far as the poly(A) tail of ET mRNA. The bottom panel in Figure 5(A) shows that there is an increase in the amount of ET mRNA between day 17 of gestation [day −5 in Figure 5(A)] and birth (day 0 in Figure 5A). However, it is difficult to load equal amounts of mRNA on the gel, so we normalized the ET message to that of β-actin, which is often used as a control message in this type of study. It has been shown that β-actin, which belongs to the class of growth-related immediate-early and delayed-early genes, is highly expressed in liver between day 17 of gestation and 2 days after birth. During this period the amount of β-actin mRNA stays constant [32]. When normalized to the amount of β-actin mRNA, the amount of ET mRNA increased gradually between days 17 and 22 of gestation (5–6-

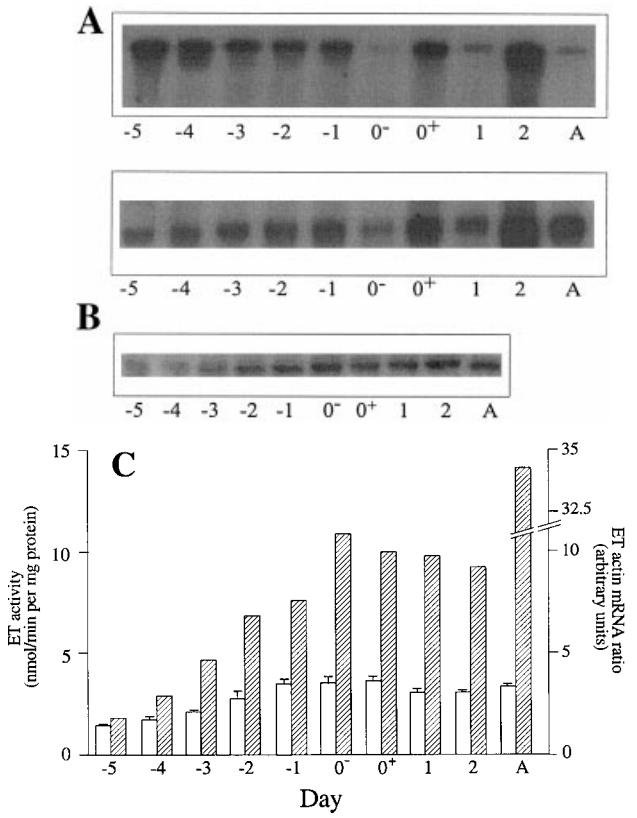

Figure 5 Rat ET activity, amount of protein and gene expression during rat liver development

Livers from prenatal and post-natal rats and from adult rats were isolated and divided into two parts. (**A**) One part was used for the isolation of mRNA. Samples (1 μg) of mRNA were separated on a gel, blotted and hybridized with a ^{32}P-labelled 1.1 kb ET-cDNA probe (lower panel) and, after stripping, reprobed with a ^{32}P-labelled β-actin probe (upper panel). ET and β-actin cDNA bands were quantified by phosphorimaging; the ratio of ET to β-actin was calculated [shaded bars in (**C**)]. The other part of the isolated liver was subjected to ultracentrifugation. The cytosolic fraction was used to determine the amount of ET protein by immunoblotting (**B**) and to assay the ET activity [(**C**), open bars]. Values are means $\pm$ S.D. for four samples, each assayed in triplicate. Days: -5 to -1, days 17–21 of gestation; 0^-, just before delivery; 0^+, immediately after delivery; 1 and 2, days 1 and 2 after birth. Abbreviation: A, adult.

fold). From then onwards, the relative ET mRNA levels stayed constant until 2 days after birth. However, in the adult liver the ratio of ET to β-actin mRNA was approx. 20-fold that in the fetal liver from day 17 of gestation. This was due to a marked decrease in the amount of β-actin message, as has been reported previously [32], whereas the level of ET mRNA stayed fairly constant between day 2 after birth and the adult state, as has been shown for protein disulphide isomerase [33]. The simultaneous increase in ET activity and mRNA level suggests regulation at a pretranslational level. However, it is clear from Figure 5 that the increase in the relative (and absolute) amounts of ET mRNA is much larger than that in ET activity. This suggests that in the developing liver ET is regulated at both a pretranslational and post-translational level.

DISCUSSION

Until now, the ET cDNA sequences of only two species, yeast [8] and human [9], have been reported. In this paper we describe the cloning of rat liver ET cDNA. Furthermore, we postulate that ET might be regulated at the level of mRNA synthesis during the development of rat liver. The identity of the clone as cDNA coding for ET is based on the following observations. (1) The amino acid sequence deduced from the rat cDNA sequence was almost completely similar to the sequence of human ET across the entire length of the protein. (2) The rat sequence also showed extensive similarity to the sequences of other cytidylyltransferases, including yeast ET, rat CT and *B. subtilis* GCT. (3) The sequences of three tryptic peptides from purified rat ET protein were found in the translation product of the cloned cDNA. (4) Transfection of COS cells with an expression vector containing the cloned cDNA as an insert caused an 11-fold increase in the activity of ET. (5) After the transfection of COS cells with the cloned cDNA, enhanced levels of a protein with the same molecular mass as purified ET and recognized by anti-ET antiserum were detected on a Western blot.

Interestingly, like human and yeast ET, rat ET has a large repetitive sequence in its N-terminal and C-terminal halves. Both parts of the repetitive sequence contain the HXGH motif, which is also present in the most conserved region in the N-terminus of members of the cytidylyltransferase superfamily [10] and class I aminoacyl-tRNA synthetases [34]. Because this motif is thought to bear the active site of these proteins [35,36], two catalytic domains can be predicted for ET. This is in line with earlier observations from kinetic studies with purified rat liver ET [37]. In these studies, two K_m values for the binding of phosphoethanolamine to ET were found, suggesting the existence of two binding sites for phosphoethanolamine.

In addition to the above-mentioned HXGH motif, the sequence of rat ET has other important characteristics that are shared by all members of the cytidylyltransferase superfamily [35]. Asp-32, which is absolutely conserved in all cytidylyltransferase sequences, is present. Further, another Asp residue (residue 241) lies very close to the HXGH motif in the second half of the repeat (Figure 2B). In addition, the sequence RTQGVSTT, which was considered a signature sequence for the cytidylyltransferase family [35], is present in rat ET. Thus rat ET seems to be a real member of this family.

The hydropathy profile of ET showed that the overall amino acid sequence is predominantly hydrophilic. The proportion of hydrophobic amino acids calculated from the rat ET sequence is 31%. This value is different from the 50% hydrophobicity reported by Vermeulen et al. [6] because in the present study glycine and alanine were not considered hydrophobic amino acids. There was no prediction of either hydrophobic regions long enough to span a lipid bilayer or amphipathic helical structures (Figure 3). This is expected for a protein of which the activity is measured only in the cytosolic fraction of the cell.

More importantly, immunogold electron microscopy studies showed that ET is enriched in areas of the cell rich in rough endoplasmic reticulum (ER), where it is located in the cytosolic space and is associated directly with the ER membrane [38]. Min-Seok et al. [8] also provided preliminary evidence that yeast ET might be associated with the ER membrane, despite the fact that the overall sequence of yeast ET protein is hydrophilic and does not contain an amphipathic α-helix. Because rat ET also lacks an amphipathic α-helical domain and has only a few very short hydrophobic stretches not longer than four amino acids, interaction of the enzyme protein with the lipid part of the ER membrane is unlikely. It is tempting to speculate that ET associates with the ER membrane through protein–protein interaction with one of the proteins in that membrane, e.g. ethanolamine phosphotransferase, permitting the efficient channelling of intermediates involved in the CDP-Etn pathway for PtdEtn biosynthesis [39]. However, the mechanism responsible

for the putative weak interaction of ET with the ER membrane or ER-membrane protein remains to be elucidated.

A search in the Prosite database revealed that there are two potential phosphorylation sites in rat ET, one for protein kinase C and one for casein kinase 2. To our knowledge, no phosphorylation of ET *in vivo* or *in vitro* has yet been reported. The potential phosphorylation sites in ET reside in its N-terminal domain and it will be interesting to study whether phosphorylation occurs and has a role in the regulation of ET activity *in vivo*.

In comparison with the human ET sequence, rat ET has an 18-residue insertion in the central domain between the two halves of the internal repeat. Because this peptide sequence is not conserved between the different species, it is not essential for catalytic activity. It might be involved in the specific regulation of rat ET, but further structure–function studies are required to obtain information about the meaning of the insert.

Taken together, the structure of the predicted ET protein can be divided into four regions: (1) a catalytic domain in the N-terminal half indicated by the high degree of similarity to the catalytic domain in the N-terminus of other cytidylyltransferases; (2) a central region containing a rat-specific peptide sequence; (3) a second catalytic domain in the C-terminal half that is very similar to the N-terminal half; and (4) a C-terminal end that shows almost 100% identity with the C-terminus of human ET but only weak similarity to other cytidylyltransferases.

So far there are no examples of regulation of PtdEtn synthesis or ET activity at the level of gene expression. In this study we found that ET might be regulated at the level of mRNA expression during the development of rat liver. This suggestion is based on the fact that the amount of ET mRNA, as expressed relative to the abundance of β-actin mRNA, increased during development and that it was approx. 5–6-fold higher in 2 days postnatal liver than in fetal liver at day 17 of gestation. However, how this enhanced ET mRNA level influences ET activity remains unclear because the changes in ET activity did not completely reflect those in ET mRNA levels. This discrepancy suggests that, in developing rat liver, ET activity is regulated both pre-translationally and post-translationally. In addition, with CT there are only a few examples of regulation at a pre-translational level [16–18], whereas it has been shown for CT that in both colony-stimulating-factor-1-dependent BAC1.2F5 cells [17] and in maturing type II cells [18] the increase in CT mRNA levels is the result of stabilization of pre-existing mRNA rather than increased gene transcription; the mechanism for the increase in ET mRNA remains to be elucidated.

The molecular cloning of rat ET cDNA as described in this study is a first step in elucidating the relationship between the structure and the function of ET. The availability of the ET sequence will permit the future characterization of the protein and the way in which its activity is regulated. For instance, it will be interesting to discover whether ET, like CT, is able to associate with membranes or whether the activity can be regulated by phosphorylation/dephosphorylation events. Furthermore, the ET cDNA will provide tools that can be used to gain insight into the regulation and the physiological importance of the CDP-Etn pathway for PtdEtn synthesis.

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